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PHOTOCHEMISTRY OF VISUAL PURPLE.

I. The Kinetics of the Decomposition of Visual Purple by Light.

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(Received for publication, July 24, 1920.)

I.

An analysis of the progress of retinal dark adaptation in man has shown that the process follows the course of a bimolecular reaction (Hecht, 1919–20). The interpretation to be placed on these findings is that dark adaptation depends on the disappearance in the retina of *two* products of decomposition resulting from the photolysis of a sensitive material. This rests on the assumption that the sensitivity of the peripheral retina measured in terms of the threshold stimulus is directly proportional to the quantity of residual decomposition products then present in the retina.

A photosensitive substance undoubtedly exists in the irritable elements, and its decomposition by light must form the first step in the receptor process. It may be supposed that this sensitive substance breaks down into two products of decomposition; and that in the dark these two products reunite to form the sensitive material again. This reversible reaction may be expressed as

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A$$

in which S is the sensitive substance, P is the principal precursor decomposition product, and A its accessory. Such a scheme has been shown to fit the facts of retinal adaptation, and to offer a starting point for quantitative investigations into the mechanism of visual reception (Hecht, 1919–20).

In terms of such an hypothesis of photoreception it would be a distinct step forward to be able to identify the three components of the reaction, and to discover their exact chemical interrelations. At present no definite statement is possible in this connection. However, dark adaptation is decidedly a phenomenon of dim vision. According to the Duplicity Theory, dim vision is associated with the rods of the retina. The rods contain a photosensitive substance known as visual purple. It is therefore *possible* that dim vision and, more particularly, dark adaptation are in some way conditioned by the properties of visual purple.

The association of visual purple with dim vision rests on more than the mere possibility just stated. A comparison of the threshold of visual sensibility at different wave-lengths with the spectral absorption of visual purple and its photochemical sensitivity has shown that these three sets of data resemble each other to a striking degree (Trendelenburg, 1911). It is similarly with the relation between dark adaptation and visual purple. A retina exposed to light is bleached. Placed in the dark, the intact eye or the retina alone with its pigment layer will regenerate the purple color. Under similar conditions the living eye becomes dark-adapted. The presumption is therefore that these phenomena are in some way correlated.

Our judgment in this matter must, however, be withheld, because no quantitative data are available with regard to the photochemistry of visual purple. Ever since Boll's (1876) discovery of this substance, and Kühne's classic qualitative investigations with it (Ewald and Kühne, 1878), there have been but a few scattered observations made with visual purple. A notable exception is the work of Trendelenburg (1904). It, however, has been barely a beginning.

It has therefore been necessary to investigate the visual purple mechanism from the modern point of view of photochemistry, and to attempt a complete analysis of the reactions concerned. The present paper is the first of a series in which the results of this investigation are to be presented. As a product of these researches, I hope to be able first, to decide whether visual purple is really intimately concerned with dark adaptation and the reception of dim lights, and, second, if it is so concerned, to determine precisely the way in which this is brought about.

II.

1. Thanks to the work of Ewald and Kühne, the preparation of visual purple is a simple matter. For my purposes the following routine has been adopted. Thirty frogs are placed over night in complete darkness. The next morning the heads are removed and placed in salt solution. The eyes are then enucleated, cut in two, and the retinas removed as free from the pigment layer as possible. This is much facilitated if the frogs have been kept over night in a warm room. The retinas with some salt solution are now placed in a centrifuge tube, and centrifuged at a good speed for 30 minutes. The supernatant liquid is removed, and a 3 to 4 per cent solution of bile salts is added to the retinas. About 5 cc. are usually added, but this may be varied up to 20 cc. depending on the concentration desired. The retinas are thoroughly stirred up, and are allowed to remain in suspension for half an hour or more. The mixture is then again centrifuged. This brings the disrupted retinas and stray pigment granules to the bottom of the tube, whereas the supernatant liquid is a clear purplish pink solution of visual purple. In this form it may be kept on ice for as long as a week or more without deterioration. It can be used directly or after dilution with water or bile salts solution.

All the manipulations are carried out in the dark, with the aid of the light from a 10 watt ruby lamp. This is hardly a source of error, because the solutions are quite insensitive to such red light. However, even to this light, they are exposed as little as possible. In considering sources of error it may be well to mention the purity of the bile salts. The commercial preparations are hopeless. The bile salts which I used were prepared in our own laboratory, and are a white powder, which when dissolved in water gives an absolutely colorless solution. Only such bile salts give reliable results.

A solution of visual purple prepared in this manner is extremely sensitive to light. If exposed to sunlight it loses its color in about a second. The solution, however, is not bleached to a completely colorless condition. The bleached solution is slightly but definitely yellow, and remains so no matter how long the exposure is maintained. In this I can confirm Trendelenburg (1904) as opposed to Ewald and Kühne's original statement (1878, p. 181) that the solu-

tion first turns yellow, and after some time turns completely colorless (*wasserhellig*). The discrepancy between the two observations I have not yet been able to explain.

The velocity of visual purple bleaching varies with the intensity; the rate may thus be controlled to allow a quantitative following of the process. With intensities near 100 meter candles the solutions may take half an hour to bleach. The purplish pink solution slowly loses its color, at the same time taking on a yellowish tinge, which increases and remains. The solutions obtained by the present technique do not show a reversible reaction. The bleached solutions retain their yellowish color in the dark for as long as they can be kept from bacterial decomposition. The conditions under which reversal takes place are different in several respects, and will be considered at length in future contributions.

2. The most obvious means of following the process is a colorimetric one. Unfortunately only a small quantity of material is available, and in dilute solution at that. A method has therefore been devised by means of which small amounts of solutions are manipulated accurately, and are made to produce the same optical effects as large quantities. The experiments are performed in very small test-tubes, made by sealing one end of short lengths of narrow bore glass tubing. The tubes are about 50 mm. long, and have an inside diameter of 2 mm., and an outside diameter of 2.5 mm. Each test-tube holds a little over 0.1 cc. This quantity of solution produces a depth of nearly 35 mm. Viewed end-on, the effect is produced of a solution of much greater concentration.

The crucial point is, of course, to have equal depths of liquid in all tubes of any given set of experiments. This is accomplished very simply. The test-tube is placed into a wider tube which fits it snugly. One end is sealed, and near the open end is a circular scratch. The inner tube rests on the bottom of the measuring tube. The solution is now inserted into the inner tube by means of a pipette having a long capillary tip, and the depth of the solution is so adjusted that the bottom of the meniscus is on a line with the circular scratch. This can easily be done within 0.2 mm., and, including slight variations in the thickness of the sealed end of the test-tube, would make the error in this part of the manipulation of the order of 1 per cent.

3. In order to follow quantitatively the progress of the bleaching reaction, it is necessary to have a series of colorimetric standards representing different concentrations of visual purple and its decomposition products. Eleven such concentrations are made up, representing on the one extreme 100 per cent of the unbleached substance plus 0 per cent of the bleached; and on the other extreme, 0 per cent of the unbleached plus 100 per cent of the bleached, the concentrations changing by 10 per cent steps. Actually these are made up in dry test-tubes by taking a total of 20 drops of solution: the first tube having 20 of the unbleached; the next tube 18 of the unbleached plus 2 of the bleached; the next 16 of the unbleached plus 4 of the bleached, etc. The same dropping pipette is used each time to insure uniformity.

The experimental unbleached solution is pipetted into the small exposure tubes. Into similar tubes and under the same conditions are pipetted the standard concentrations just described. The standards are jacketed with thick, black rubber tubing, except for a few millimeters at each end. In this way a much clearer end-on view is obtained. The exposure tube containing the experimental solution of visual purple is similarly jacketed when colorimetric comparisons are to be made. The matching is done against an artificial daylight lamp of 0.04 candle power, having a uniform circular surface 30 mm. in diameter. Such a light is sufficiently bright for work in the dark, especially after one has become dark-adapted. The matching light is turned on and off by a spring contact controlled by the observer's foot, so that only a momentary exposure is made.

When not in actual use the standards are kept under cover even in the dark room. As a rule, fresh standards are made daily. If they have been used but little, they may be kept on ice until the next day, provided they are placed in a moist atmosphere so as to prevent evaporation. They suffer comparatively little deterioration from their use, mainly because the exposures to the daylight lamp are so brief. After continuous use for several days, they are definitely off; hence the practice of making fresh standards daily.

4. The experimental tube is exposed to light of known intensity. The apparatus used for this purpose is shown full size in Fig. 1. The glass rod to which the exposure tube is attached is rotated by hand at a rate depending on the intensity of the light. For the experi-

ments reported in this paper this was roughly ten revolutions per minute. By this means an even exposure is obtained for this thin column of solution. This obviates the necessity for stirring the solu-

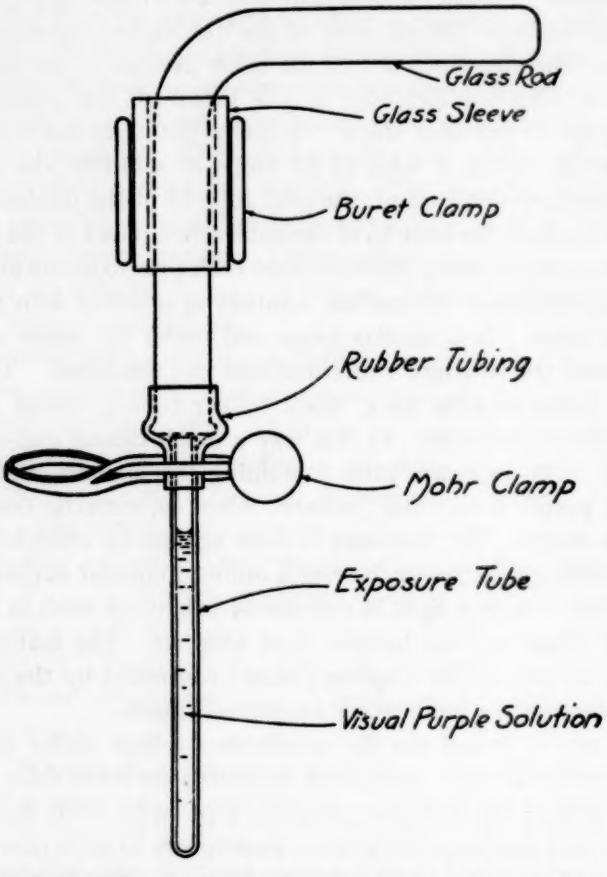

FIG. 1. Full size diagram of apparatus used for the exposure of visual purple solutions to light. The exposure tube being attached to the glass rod, it is possible to rotate the tube on its own axis by rotating the glass rod.

tion, and removes all errors associated with unequal illumination, diffusion, etc. (*cf.* Sheppard, 1914, p. 239). In this respect the small quantities of material and the thin test-tubes are of distinct advantage. After the proper length of exposure the light is turned off, the tube

removed, jacketed with the rubber tubing, and compared with the standards.

Readings are usually made to the nearest 5 per cent concentration. The reading is thus, say, 70 or 75 per cent. Occasionally, however, it is so clear that the color is nearly but not quite one of the 10 per cent tubes, that the reading is given between the 5 and the 10 per cent

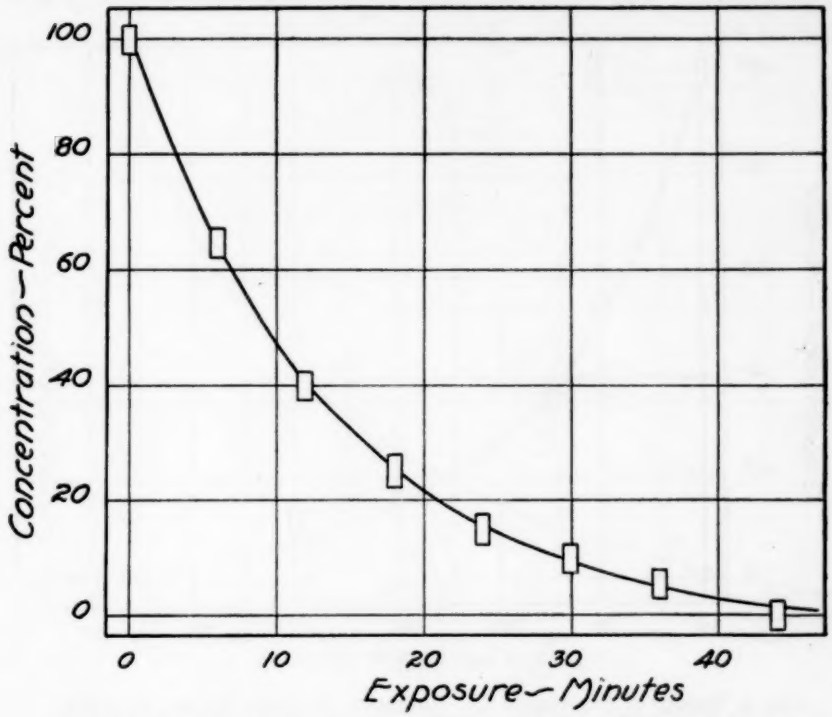

FIG. 2. The results of Experiment 80. Light intensity is 53 meter candles. The monomolecular nature of the bleaching process is evident from the coincidence of the experimental points (rectangles) with the curve calculated on the assumption that $k = 0.034$.

steps; for example, 48 or 52. This happens comparatively infrequently.

After a colorimetric determination the jacket of the tube is removed, the tube replaced, and the exposure renewed. After a sufficient exposure, the tube is again removed, and its concentration again determined. The process is repeated until the bleaching is complete.

III.

1. Altogether I have followed the progress of the bleaching of visual purple in 88 separate experiments. These have been performed under a variety of conditions to which reference will be made in the proper place. Figs. 2, 3, and 4 will serve as examples of what happens. They represent random samples of experiments performed so as to give

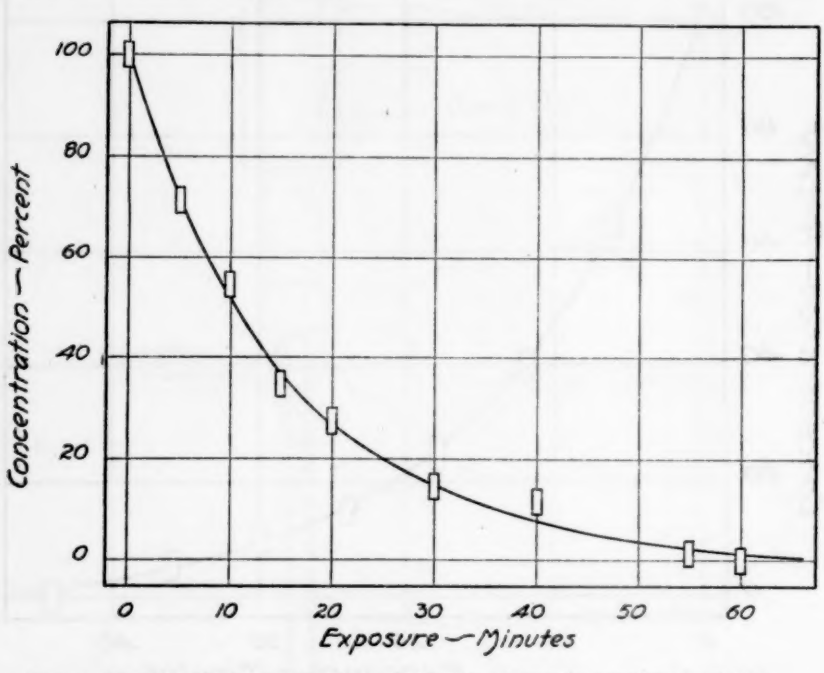

FIG. 3. Results of Experiment 42. Intensity, 28 meter candles; $k = 0.028$.

different velocities of decomposition, and are quite characteristic of all the other experiments.

In the figures the readings are plotted as rectangles, on the assumption of an error of 5 per cent in the determination of the concentration, and 1 minute in the control of the exposure. The curves are isotherms of a monomolecular reaction

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

in which a is the original 100 per cent concentration, and $a-x$ the per cent of material still unbleached. It is apparent that the experimental results follow the curves pretty well.

To show this in the usual way Table I is given presenting the results of a fourth experiment. It will be observed that the values of the velocity constant k are fairly constant. This uniformity is particularly striking if two things are kept in mind: first, that the measurements are single determinations only, not averages; and, second,

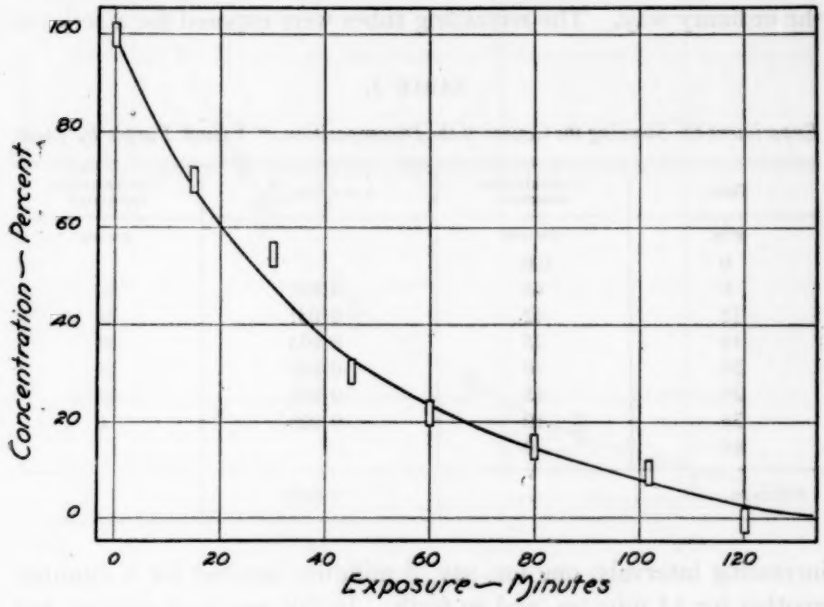

FIG. 4. Results of Experiment 56. Intensity, 12 meter candles; $k=0.011$.

that they are read usually to the nearest 5 per cent concentration. For further comparison there are inserted the values calculated from the formula assuming the average value of k ($= 0.030$). The similarity between the observed and calculated values is obvious.

2. Each of the experiments so far considered represents the changes in a single tube of visual purple. The solution is exposed for an interval, its concentration determined, then exposed for a second interval, and the process repeated until no further change occurs. Such a

mode of procedure assumes two things: one, that there is no period of induction in the photochemical action; and the other, that there is no after effect. If either of these is associated with the reaction, a different type of result,—qualitative perhaps, certainly quantitative,—is to be expected depending on whether the exposure is continuous or interrupted.

In order to settle this point several series of experiments were made in the following way. Seven or eight tubes of similar concentration and depth were prepared. One or two were run through in the ordinary way. The remaining tubes were exposed for a series of

TABLE I.

Experiment 85, Showing the Course of the Decomposition of Visual Purple by Light.

Time.	Concentration observed.	$k = \frac{1}{t} \log \frac{a}{a-x}$	Concentration calculated.
min.	*per cent*		*per cent*
0	100		
6	65	0.031	66
12	42	0.031	44
18	25	0.033	29
24	20	0.029	19
30	15	0.027	13
36	10	0.028	8
46	0		
Average.....		0.030	

increasing intervals; one for, say, 5 minutes, another for 8 minutes, another for 12 minutes, and so forth. In this way each solution was brought to a stage of decomposition by a continuous exposure as opposed to a series of intermittent exposures.

An example of such an experiment is given in Fig. 5. The black rectangles represent a single tube exposed intermittently in the ordinary way. The white rectangles each represent a separate tube of visual purple exposed continuously up to the point when its concentration was determined. The curve is again that of a monomolecular reaction, and it is apparent that it represents accurately the course of both types of experiment.

In a few tests the procedure was slightly varied. Two sets of tubes were run. In the one the readings were made intermittently, there being about seven interruptions in the exposure. In the other, the readings were also made intermittently, but with only two or three interruptions in the exposure. The results in the two cases were the

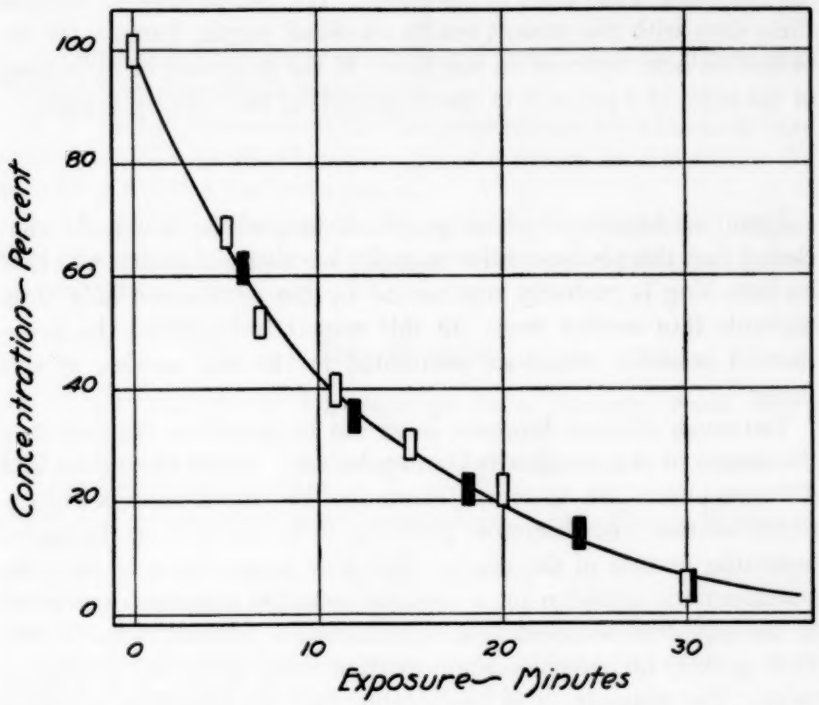

FIG. 5. A comparison of the effects of intermittent (black rectangles) and continuous (white rectangles) illumination on the velocity of decomposition. The black rectangles are from a single tube of visual purple. Each of the white rectangles represents a separate tube. Experiments 32 to 38 inclusive; intensity, 53 meter candles; $k = 0.037$.

same within the limits of the experimental error. It may therefore be safely concluded that there are no aberrant factors such as an induction period or an after effect operating in these experiments.

It will be remembered that these results are to be expected on the basis of Talbot's law for the fusion of successive light flashes. In such

experiments the rate of intermittent illumination for the human eye is very much greater than those used here. The principle, however, is the same. Parker and Patten (1912–13) have recently shown that a continuous flow of light is a more efficient stimulus for the eye than an intermittent one (1,750 flashes per minute), the difference between the two being of the order of 5 per cent. It is not possible to compare these data with the present results on visual purple, because the experiments here reported do not admit of the judgment of differences of the order of 5 per cent in the magnitude of the velocity constant.

IV.

From the kinetics of visual purple decomposition it may be concluded that this photosensitive material is a chemical entity, and that its bleaching is probably represented by the destruction of a large molecule into smaller ones. In this respect it resembles the hypothetical sensitive substance postulated in the first section of this paper.

Too much reliance, however, must not be placed on the fact that the course of the reaction is monomolecular. Gross absorption and diffusion effects are, of course, eliminated by the thinness of the exposed solution; they therefore probably do not account for the monomolecular feature of the curve. But it is frequently true that the stoichiometric equation for a chemical reaction does not correspond to the equation obtained from kinetic data. In such cases (Lewis, 1918, p. 395) the stoichiometric equation turns out to be of a higher order. For example, it is conceivable that the bleaching of visual purple is a process of hydrolysis, in which case the stoichiometric equation may actually be bimolecular. However, because there is so much water present in the system, the reaction would proceed as if it were monomolecular, visual purple being the only component whose concentration changes to a measurable degree.

The equation

$$S \rightarrow P + A$$

for the hypothetical light reaction is also not to be taken dogmatically. It may well be

$$S + X \rightarrow P + A$$

assuming that X is a substance whose concentration in the system is so great that it suffers no sensible change during the reaction. Further discussion of these matters will, however, be postponed to a time when more data will have been presented.

SUMMARY.

1. Visual purple solutions are prepared under such conditions that the bleaching reaction is irreversible.

2. A method is described for the colorimetric estimation of very small quantities of visual purple. By this means the kinetics of the bleaching reaction are investigated.

3. The results show that the course of the decomposition follows that of a monomolecular reaction, without any measurable period of induction or after effect.

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THE MECHANISM OF INJURY AND RECOVERY.

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During the process of death the electrical conductivity of many tissues undergoes a change in electrical resistance by means of which the process may be followed with considerable precision. This may be utilized to measure injury and recovery.[1]

An illustration of this is seen in Fig. 1, which shows the gradual fall in resistance[2] of *Laminaria* placed in 0.52 M NaCl.[3] After an exposure of 5.2 minutes the resistance has fallen to 94.6 per cent of the resistance it had in sea water.[4] If the tissue is then replaced in sea water the resistance rises (uppermost dotted line) and returns to practically normal value. This rise of resistance may be spoken of as recovery.

[1] Attention has been called in a previous paper (Osterhout, W. J. V., *J. Biol. Chem.*, 1915, xxiii, 67) to the need of accurate determinations of toxicity and to the use of electrical measurements for this purpose.

[2] For convenience in making comparisons the death curves used in a former paper (Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533) are employed as standards. Since in the recovery experiments the death curves vary somewhat (depending on the temperature, the condition of the material, etc.), it is permissible for comparative purposes to make the death curves coincide with the standard in as far as this can be done by multiplying the abscissæ by the proper factor (which is always the same for all the abscissæ of any particular recovery curve). This has been done throughout the experiments: otherwise an accurate comparison of the recovery curves would be difficult. The corrections made are relatively small, since, in order to make the results comparable, all experiments showing a wide deviation from the standard curves were rejected. Since the deviations are chiefly due to differences in temperature the chief result of the corrections is to make all the reactions appear as if they had taken place at the same temperature.

[3] All the solutions had the same conductivity as sea water.

[4] For convenience all results are expressed as per cent of the original resistance.

If, however, the exposure to the solution of NaCl lasts longer the tissue does not recover its normal resistance when returned to sea water. After prolonged exposure recovery is much less complete and as the tissue approaches the death point there seems to be no recovery when it is removed from the solution of NaCl and kept in sea water under normal conditions.

Fig. 1 shows that the recovery curves rise to certain definite levels and then run horizontally. If the conditions are favorable they may maintain this horizontal course for days; but if the control falls they also fall.

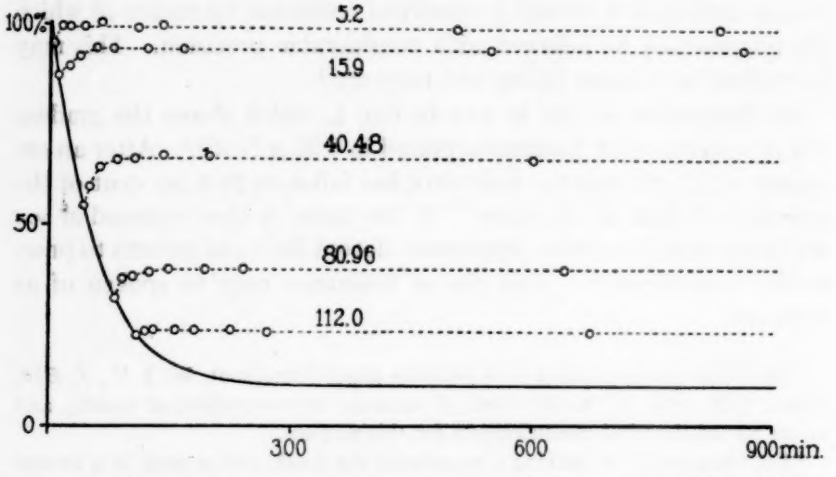

FIG. 1. Curves showing electrical resistance of *Laminaria agardhii* in NaCl, 0.52 M (unbroken line), and recovery in sea water (dotted lines). The figure attached to each recovery curve denotes the time of exposure (in minutes) to the solution of NaCl.

Another aspect of recovery is illustrated by the results obtained in mixtures of NaCl and $CaCl_2$. Curve C in Fig. 2 shows the behavior of tissue placed in a solution containing 97.56 mols of NaCl to 2.44 of $CaCl_2$; its electrical resistance falling in 37.5 hours to 72.87 per cent of the original value in sea water. In a solution containing 85 mols of NaCl to 15 mols of $CaCl_2$ (Curve A) the resistance fell in the same time to practically the same point (72.47 per cent).

When these two lots of tissue were replaced in sea water they behaved differently. The resistance of the first lot rose to 78.2 per cent

(Fig. 2, upper dotted line) but the resistance of the second fell (much more rapidly than if it had not been removed to sea water) and eventually became practically stationary at 38.1 per cent (Fig. 2, lower dotted line).

One method of explaining these facts is to assume[5] that the resistance is proportional to a substance *M* (contained within the cells), which is

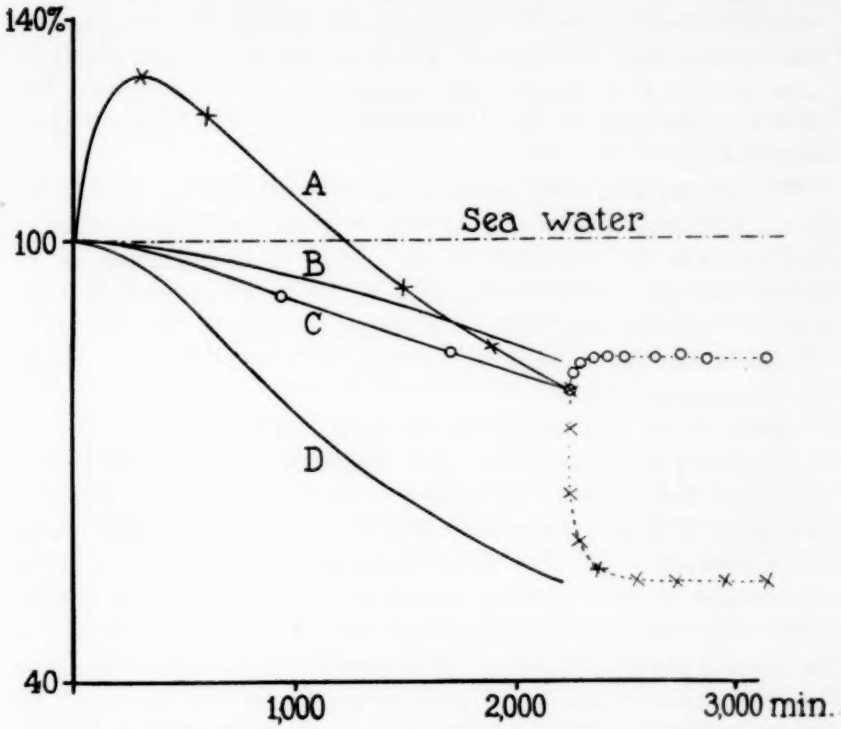

FIG. 2. Curves showing electrical resistance of *Laminaria agardhii* in a solution containing 97.56 mols of NaCl to 2.44 mols of $CaCl_2$ (Curve C) and in a solution containing 85 mols of NaCl to 15 mols of $CaCl_2$ (Curve A). The dotted lines show recovery in sea water. Curves B and D show the levels to which the resistance rises when the tissue recovers in sea water after exposure to these mixtures; their abscissæ denote the times of exposure. Curve B pertains to the first mixture (belonging with Curve C), while Curve D pertains to the second mixture (belonging with Curve A).

[5] *Cf.* Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533.

formed and decomposed by a series of reactions according to the scheme

$$O \rightarrow S \rightarrow A \rightarrow M \rightarrow B$$

We assume that in sea water the amount of M remains constant because it is formed as rapidly as it is decomposed. When, however, it is transferred to a solution of NaCl (or to the first of the two mixtures mentioned above) the resistance falls, because M is decomposed more rapidly than it is formed. In the second mixture the resistance rises, because M is formed more rapidly than it is decomposed, but after a certain time the rate of formation decreases and the resistance falls steadily.

We suppose that when tissue is placed in the solution of NaCl (or in the mixtures) the reactions $O \rightarrow S \rightarrow A$ cease. In the solution of NaCl (and in the first mixture) the amount of M diminishes on account of the gradual exhaustion of A; in the second mixture the reaction $A \rightarrow M$ is so rapid that a temporary increase in the amount of M results, but this is inevitably followed by a decrease as the supply of A becomes exhausted.

When the tissue is replaced in sea water the reactions $O \rightarrow S \rightarrow A$ are supposed to recommence. The supply of A is therefore replenished and the resistance will return to the normal value, provided O is present in large amount and suffers practically no change during the experiment. But if it should diminish by one-half as the result of exposure to NaCl it could restore M to only half of its original value. We may therefore regard the level to which M is restored as an index of the condition of O. If we plot this level after various periods of exposure to the first mixture we get Curve B in Fig. 2 (and for the second mixture, Curve D).[6]

If we use the term recovery for the rise of resistance which occurs when tissue is transferred to sea water from certain solutions (such as the first mixture) there seems to be no good reason why it should not be applied to the fall of resistance which occurs when tissue is transferred from certain other solutions (such as the second mixture) to sea water. The amount of recovery after any given period of exposure

[6] A similar curve could be drawn for Fig. 1 but it has been omitted in order to avoid confusion.

is equal to the vertical distance between Curves B and C, in the case of the first mixture, and between Curves A and D in the case of the second mixture.

If we use the term injury to denote loss of resistance, we may define temporary injury[7] as the loss which occurs in the solution and permanent injury as the loss which is observed after the tissue has been returned to sea water and the resistance has risen or fallen to a stationary condition. Curves B and D are therefore the curves of permanent injury.

It may be asked whether Curves B and D are better criteria of toxicity than Curves A and C. The question involves the definition of toxicity. Since this term is used in a variety of ways, it is desirable that it should always have a precise quantitative significance. In the present case it is evident that we need not only A and C but also B and D for a complete description of the facts. It seems possible that this may be generally true in the study of toxicity, although at present we may be unable to construct similar curves in many cases because suitable methods of measurement are lacking.

The fact that recovery is never complete except at the beginning (as shown by Curves B and D) might also be explained as due to the death of certain cells; for if some of the cells are killed by exposure to a solution of NaCl the complete recovery of the surviving cells cannot restore the resistance to its normal value. The chief objections to this explanation are that during the death process the cells seem to act alike and to die at about the same time; moreover this assumption does not lend itself readily to quantitative treatment. It does not seem to be necessary to discuss this point more fully at present; but it may be pointed out that this hypothesis would in no way invalidate the conception developed above, that an individual cell may lose part of its resistance and subsequently regain it, either partially or completely.

[7] Substances which cause increase of resistance commonly produce permanent injury; this is apparent when the tissues are replaced in sea water. It would therefore seem that any alteration of resistance (increase or decrease) may produce permanent injury if sufficiently prolonged. In spite of this it seems preferable to restrict the term temporary injury to the fall of resistance observed in toxic solutions without coining a new term to express the injurious action of rise of resistance.

The fact that in the case of *Laminaria* recovery may be either partial or complete, according to circumstances, raises the question whether this is also true of other forms. It is certainly true of all the plants investigated by the writer, such as the green alga, *Ulva* (sea lettuce), the red alga, *Rhodymenia* (dulse), and the flowering plant, *Zostera* (eel grass). It seems to be also true of frog skin as far as the experiments of the writer have gone.[8] In physiological literature it seems to be generally assumed that when recovery occurs at all it is practically complete. It is evident that partial recovery might easily be overlooked except in cases where recovery can be measured with considerable accuracy, and it seems possible that further investigation may show that partial recovery is a general phenomenon.

If we accept the conclusions stated above we are obliged to look upon recovery in a somewhat different fashion from that which is customary. Recovery is usually regarded as due to the reversal of the reaction which produces injury. The conception of the writer is fundamentally different; it assumes that the reactions involved are irreversible (or practically so) and that injury and recovery differ only in the relative speed at which certain reactions take place.

Thus in the series of reactions $O \rightarrow S \rightarrow A \rightarrow M \rightarrow B$, if the rate of $O \rightarrow S$ becomes slower than the normal, injury will occur, while a return to the normal rate will result in recovery. Injury could also be produced by increasing the rate of $M \rightarrow B$, or decreasing the rate of $S \rightarrow A$ or $A \rightarrow M$.

If life is dependent upon a series of reactions which normally proceed at rates bearing a definite relation to each other, it is clear that a disturbance of these rate-relations may have profound effects upon the organism, and may produce such diverse phenomena as stimulation, development, injury, and death. It is evident that such a disturbance might be produced by changes in temperature[9] (in case the temperature coefficients of the reactions differ) or by chemical agents. The same result might be brought about by physical means, especially where structural changes occur which alter the permeability of the plasma membrane or of internal structures (such as the nucleus and plastids) in such a way as to bring together substances which do not normally interact.[10]

[8] The recovery experiments on frog skin have been few in number and were devoted chiefly to the effects of anesthetics.

[9] *Cf.* Osterhout, W. J. V., *J. Biol. Chem.*, 1917, xxxii, 23.

[10] Or which normally react to a lesser degree.

INHIBITORY ACTION OF PARATYPHOID BACILLI ON THE FERMENTATION OF LACTOSE BY BACILLUS COLI. I.

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In nature, organic matter is attacked by a large variety of bacteria and in its disintegration a number of species may take part. A knowledge of just how the species are associated in any given substrate or in what order they succeed one another in the process of decomposition might be of great value to the biochemist in supplying a point of approach hitherto neglected. There is evidence that there is some order in the mode of attack and that this order is conditioned by the nature of the medium and certain environmental factors, such as temperature, reaction, and oxygen supply. In the following pages one single detail of this general problem was examined under conditions which excluded all but two bacteria, one following the other.

If a fermentation tube containing fermented bouillon plus 1 per cent lactose be inoculated with some member of the paratyphoid group, the multiplication causes a heavy clouding or even turbidity in the open arm.

The growth in the closed arm depends on the degree to which the muscle sugar has been removed. If this is complete, no growth takes place. The clouding of the branch is therefore proportional to the amount of muscle sugar remaining. Titration shows little or no change of reaction in the closed arm, beyond what is due to a movement of fluid from open to closed arm. The fluid in the open arm becomes neutral and finally alkaline to phenolphthalein, the degree of alkalinity being a function of the multiplication which in turn is a function of the alkali-tolerance of the particular strain used. If after 4 to 6 days of incubation the same tube is reinoculated, this time with a true colon bacillus known to ferment lactose, one of two things may happen:

1. The resulting fermentation takes the usual course as if no prior inoculation had been made; *i.e.*, the production of acid and gas goes on quantitatively as in a fresh tube.

2. The second organism multiplies actively, clouds the closed branch as usual, but no gas is produced. Titration shows that the usual amount of acid has been produced.

In order to determine whether the particular type of second or superadded fermentation is constant for the same organism, numerous tests have been made during the past 1½ years, with the result that with the same combination of cultures the same result follows.

Furthermore the various types of paratyphoid bacilli which happened to be on hand in the laboratory were tested to see just what grouping would result if this kind of superadded fermentation were used as a basis of classification. The results of this latter test are in many respects suggestive.

All true hog-cholera bacilli left the lactose medium uninfluenced. The fermentation of the second organism proceeded as in a fresh unused medium. Among the hog-cholera bacilli were those of widely different ages. The oldest one had been isolated by one of us early in 1886[1] and was described at that time as a membrane-producing variant. The most recent strain had been isolated by Dr. TenBroeck at Camp Upton late in 1918 (No. XVI).

Among cultures not isolated from swine, *Bacillus icteroides* Sanarelli acted like a true hog-cholera bacillus. That this organism may have been a true hog-cholera bacillus is made highly probable by the experiments of Reed and Carroll.[2] They found a complete identity of the two types in morphological, cultural, pathogenic, and agglutinative characters. *Bacillus icteroides* also produced fatal enteritis associated with necrosis of the mucosa of the large intestine in young pigs. One of these experiments was so carefully controlled that the, at that time, still unknown filterable virus of hog-cholera could hardly have come into consideration as a cause of the lesions. Moreover the autopsy notes do not suggest the filterable virus.

[1] Smith, T., *3rd Ann. Rep. Bureau Animal Industry, U. S. Dept. Agric.*, 1886, 622.

[2] Reed, W., and Carroll, J., *J. Exp. Med.*, 1900–01, v, 215.

Of the cultures isolated from swine which failed to give the same reaction as the true hog-cholera bacillus was a non-motile organism isolated by Professor T. J. Burrill from the liver of a pig in 1897, and described briefly by one of us[3] in 1899. This strain could be differentiated from the true hog-cholera bacillus only through the absence of motility and a reduced virulence. *Bacillus coli* following this produced only a large bubble of gas in lactose bouillon.

Among the cultures tried which belong to the first group are Hog-cholera Nebraska (1886), Massachusetts (1895), Maryland (1898), Arkansas (1899), and Hog-cholera X, XI, XII, XIII, XIV, and XVI, each from a different outbreak; also *suipestifer* Voldagsen, and the non-gas-producing hog-cholera strain of M. Dorset. In several of the above strains mutants had appeared in the stock cultures. One of these produced a viscid growth, the other had lost the power of gas production in lactose. All these acted alike in not restraining gas production subsequently by *Bacillus coli*.

Among the paratyphoid and *enteriditis* strains in the collection the following came under the second group: paratyphoid strains recently isolated from four calves and one fetus by one of us; paratyphoid Strains 225, 232, 234, and 242; Schottmüller A and B; paratyphoid from a pigeon and a cow's brain, all received from other laboratories; Rat-typhus I, III, IV, and V; Swine-typhus I, II, III, IV, and V, Spermophile typhus I, Dog-typhus I, Guinea pig-typhus VII, VIII, Mouse-typhus I, II, *Bacillus enteriditis* 47, 204, Gaertner (Kral and Mt. Sinai), and swine *enteriditis*. The one exception noted thus far was marked paratyphoid (Longcope). Dr. TenBroeck[4] had previously examined this strain and found it culturally and serologically a hog-cholera bacillus. Its virulence, however, towards rabbits is very low as compared with true hog-cholera bacilli.

One culture of human typhoid tried thus far inhibited gas production and belongs therefore to the second group. A culture of paratyphoid bacilli from man which Hirschfeld has denominated Group C and which has been shown by TenBroeck[4] to belong culturally to the paratyphoid and serologically to the hog-cholera group, but with

[3] Smith, T., *Centr. Bakt., Ite Abt.*, 1899, xxv, 241.
[4] TenBroeck, C., *J. Exp. Med.*, 1920, xxxii, 33.

a reduced virulence, acted like a true paratyphoid in inhibiting gas production.

The rather sharp distinction which this reaction makes between true hog-cholera bacilli and other paratyphoid strains made it seem desirable to test the several members of the dysentery group on hand. All strains tested, including those marked Shiga, Flexner, Hiss Y, Flexner-Harris, and Strong, acted like the hog-cholera group in that the second or *Bacillus coli* fermentation was not inhibited as to gas production. Similarly the group of paratyphoid-like bacilli attacking poultry (fowl typhoid and white diarrhea) did not inhibit gas production by *Bacillus coli*.

So far, to avoid confusion, the second fermentation has been restricted to two colon strains isolated from a calf. The strain of *Bacillus coli* used most constantly in the various experiments was isolated in December, 1918, from the liver of a calf (No. 302) affected with pneumonia and septic complications. It possesses motility to a certain degree, ferments dextrose and lactose with the accumulation of from 50 to 60 per cent gas in the closed arm of the fermentation tube, and produces a titratable acidity of 4 to 6 per cent of a normal solution. The gas formula H/CO_2 is of the usual type, the explosive portion being 2 to $2\frac{1}{2}$ times the CO_2 in volume. Saccharose is fermented more slowly and about 25 per cent gas accumulates. The second strain (No. 435) was isolated from the spleen of a very young calf affected with digestive derangements, called scours. It was in all cultural characters like the preceding.

Wherever gas production is referred to in the following pages, the accumulation of gas in the fermentation tube is meant. The acid produced is quoted in per cent of titratable acid in terms of a normal solution.

It is to be predicted that all races of *Bacillus coli* will not act alike since there are so many minor distinguishing morphological, cultural, fermentative, and serological features observed among them. Some preliminary tests with other colon bacilli have been made but the data are incomplete.

Experiments to Determine the Nature of the Inhibition.

The Effect of Removing the Paratyphoid Bacilli.—The first suggestion to present itself was that the paratyphoid bacilli might have changed the lactose in some way. If so, in the bouillon deprived of the paratyphoid bacilli by filtration, the colon bacilli should act differently than in fresh bouillon. It was found, however, that the complete removal of paratyphoid bacilli from the culture fluid by passing it through a Berkefeld filter restores gas production. The experiments were made by growing the paratyphoid bacilli in large centrifuge bottles and filtering after centrifuging. The filtrate was put into sterile fermentation tubes and inoculated with *Bacillus coli*. Table I gives some of the results.

TABLE I.

Strain of paratyphoid used.	Age of culture when centrifuged and filtered.	Results of inoculation with *B. coli*, Calf 302.	
		Gas.	Acid in open arm.
	days	*per cent*	
Calf 299	10	26.5	5.10
" 299	10	45.0	4.75
" 297	6	47.2	4.60
" 297	6	52.0	

The next step was to proceed as in the former experiment but to omit the filtration. The centrifuged fluid did not become entirely clear and when inoculated with *Bacillus coli* only a very little gas was produced. Apparently the presence of a very faint cloud of paratyphoid bacilli was sufficient to inhibit almost completely gas production by *Bacillus coli*. The hog-cholera control treated in the same way did not inhibit gas production (Table II).

In order to remove as far as possible all bacteria without subjecting the culture fluid to final filtration, the following procedure was carried out. The primary culture was conducted as in the foregoing experiment in large centrifuge bottles, holding 250 cc. and plugged with cotton wool. After an incubation of about 4 days sufficient to yield a maximum multiplication a certain quantity of sterile kaolin powder was added to the fluid, the bottles were closed with

sterile rubber stoppers, and thoroughly shaken by hand. They were then centrifuged for about 30 minutes, at the end of which time the supernatant fluid was clear to the unaided eye in transmitted light. It was then transferred with sterile pipettes to sterile fermentation tubes and inoculated with *Bacillus coli*, Calf 302.

TABLE II.

Strain used.	Age of culture when treated.	Time of centrifugation.	Results of inoculation with *B. coli*, Calf 302.	
			Gas.	Acid in open arm.
	days	*min.*	*per cent*	
Paratyphoid, Calf 297.....	7	30	Bubble only.	3.80
" " 297.....	7	30	1–2	4.61
Hog-cholera XII.....	10	30	60	5.30

To determine whether the kaolin itself might add anything to the medium to influence the result various controls were introduced as shown in Table III.

TABLE III.

Effect of Centrifuging and Clearing First Culture with Kaolin on Second Culture.

Experiment.	Treatment of first culture. (Paratyphoid, Calf 299.)	Results of inoculation of second culture. (*B. coli*, Calf 302.)	
		Gas.	Reaction of open arm (acidity).
		per cent	
a.....	Sterilized kaolin added, bottle shaken and centrifuged 30 min., transferred to fermentation tubes.	65	4.56
b.....	Sterile control fluid, treated as under *a* before inoculating with *B. coli*.	93	4.55
c.....	Sterile control fluid without kaolin.	62	5.40
d.....	Fermented bouillon without lactose, shaken with kaolin.	0	0.27 (Open arm.) 1.58 (Closed ")

Tube *a* shows that a complete clearing of the culture fluid from paratyphoid bacilli restores gas production by *Bacillus coli* and is equivalent to filtration. Tubes *b*, *c*, and *d* were to show whether kaolin modified the fermentation. The larger amount of gas produced in *b* indicates the presence of some favoring impurity on gas produc-

tion, possibly a carbonate. Tube *c* checked the culture medium and Tube *d* proved the absence of fermentescible sugars in the kaolin.

Effect of Density of Suspension of Paratyphoid Bacilli.—In the foregoing tests the primary culture was usually 4 days old before the second inoculation was made. The mere presence of paratyphoid bacilli in the culture fluid might have some influence irrespective of any incubation period. Experiments were therefore undertaken to eliminate this period. The growth of paratyphoid bacilli on slanted agar was washed off with bouillon and suspended in lactose bouillon in fermentation tubes. The suspension was varied in density by using the growth of one, two, and four slants respectively. Immediately after the suspension was made a loopful of *Bacillus coli* was introduced from an agar culture and the tubes incubated. The gas production was not inhibited but it accumulated more slowly than in control tubes. After 7 days incubation 70, 68, and 66 per cent of gas had accumulated. The reaction of fluid in the bulb was 4.61, 4.96, and 4.91 per cent of a normal acid respectively. It should be stated that the density of the suspension of paratyphoid bacilli, even in the closed arm of the first tube, was much greater than that developing directly in lactose bouillon. No inhibition of gas production was thus produced by the mere presence of large numbers of living paratyphoid bacilli, both strains being introduced together.

In another experiment, the relation of the bacilli to the inhibition was put to a more rigorous test. A large centrifuge bottle containing lactose bouillon and inoculated with paratyphoid, Calf 297, was incubated for 6 days. One fermentation tube was then filled with the culture fluid and inoculated with *Bacillus coli*, Calf 302, and incubated as a control. Another lot from the same bottle was centrifuged until the supernatant fluid was only faintly clouded. This fluid was transferred to a second fermentation tube and inoculated with *Bacillus coli*. The thick bacterial sediment in the centrifuge tubes from 30 cc. of the culture fluid was transferred to 25 cc. of fresh lactose bouillon in a fermentation tube and inoculated with *Bacillus coli*. Over 50 per cent gas was promptly produced in the third tube. In the others a small bubble appeared partly derived from fine bubbles transferred with the culture fluid.

The Effect of the Concentration of Lactose.—In the foregoing tests 1 per cent lactose was used throughout. It was thought, however, that less or more might affect the uniformity of results. Before taking up the experiment it became necessary to consider what minimum per cent of lactose was necessary for *Bacillus coli* to produce the maximum amount of gas. Earlier, repeated trials by one of us had shown that 0.5 per cent lactose yields about as much gas as 1 per cent or more. Table IV shows that the same result is obtained whether a concentration as low as 0.5 per cent or as high as 2 per cent lactose is used. Numerous tests of the gas formula in these experiments indicated no appreciable change in the relative amounts of H and CO_2. These tests have therefore been omitted from the tables.

TABLE IV.

Effect of Different Concentrations of Lactose on Secondary Fermentation by B. coli, Calf 302.

Primary culture.	Lactose.	Interval between primary and secondary cultures.	Gas produced.	Acid produced.	
				Open arm.	Closed arm.
	per cent	*days*	*per cent*		
Paratyphoid 297	0.5	6	0	5.0	5.0
Hog-cholera XII	0.5	6	57	5.2	
Paratyphoid 297	2.0	4	0	3.7	4.8
Hog-cholera XII	2.0	4	62	4.8	

The absence of lactose in the primary culture does not interfere with the establishment of inhibition. Cultures of paratyphoid bacilli in fermented bouillon without lactose will inhibit subsequent gas production by *Bacillus coli* when the latter and lactose are added at the same time after the primary culture has grown 4 or more days. To ensure the above results, there must be distinct clouding of the closed arm by the paratyphoid bacilli. Absence of multiplication due to a too thorough removal of muscle sugar fails to establish inhibition.

Effect of the Age of the Primary Culture.—Certain experiments had indicated that the age of the primary culture before inoculation with *Bacillus coli* was not an indifferent factor. Taking the combination

paratyphoid, Calf 297, and *Bacillus coli*, Calf 302, *Bacillus coli* was inoculated at the same time with, and hours after, the paratyphoid bacillus to determine how long a growth period is required by the primary culture before it becomes completely inhibitory.

Table V shows that inhibition begins after 4 hours and is nearly complete at 14 hours with one lot of bouillon. With another it is not complete at 72 hours. This difference is probably due to the degree of removal of the last traces of muscle sugar during the prep-

TABLE V.

Effect of Age of Primary Culture on Inhibition of Gas Production.

Age of primary culture before second inoculation.	Amount of gas produced.	Acid produced in open arm.	Acid produced in closed arm.
	Bouillon 1,003.		
	per cent		
0 (Simultaneous.)	58	4.2	
2 hrs.	53	3.9	
4½ "	28	3.9	4.0
6½ "	24	4.2	3.9
8 "	20	3.8	4.2
10 "	11.5	4.4	4.2
12 "	10	4.9	4.2
14 "	5	4.6	4.9
	Bouillon 985.		
21 hrs.	13	6.0	4.7
2 days.	12.5	4.9	4.7
3 "	10	5.3	3.8
4 "	0	4.2	3.9

aration of the fermented (lactose) bouillon which controls the clouding of the closed arm.

It has been observed in certain experiments in which the second organism was introduced later than 4 days after the first, that in those cultures in which gas production was not inhibited, as in the hog-cholera group, the total quantity of gas produced was nevertheless not up to the usual amount. This observation led to the inference that perhaps here also the time element was a determining factor.

Table VI gives the results of an experiment to clear up this question. Hog-cholera bacilli were introduced into a number of lactose tubes and kept different periods of time before *Bacillus coli* was introduced. Cultures incubated 19 days before the second inoculation inhibited gas production completely. Those incubated up to 6 days failed to inhibit. The rest produced variable amounts of gas.

The paratyphoid bacillus thus produces a condition in 2 to 4 days which the hog-cholera bacillus brings about in 18 days. It is interesting to note that this period roughly corresponds to the time

TABLE VI.

Effect of Age of Primary Culture on Inhibition of Gas Production. (Primary Culture, Hog-Cholera Bacillus XII; Secondary Culture, B. coli, Calf 302.)

Age of primary culture when *B. coli* inoculated.	Amount of gas produced.	Acid produced.	
		Open arm.	Closed arm.
	per cent		
0 (Control.)	65	5.0	4.9
3 days.	60	5.2	5.7
6 "	50	6.2	5.6
11 " *	5	5.9	5.6
11 "	32	4.6	5.2
14 "	7	4.8	5.5
18 "	0	4.8	6.0
19 " *	0	4.9	5.0

* Primary culture grown with about $\frac{1}{5}$ of closed arm containing air. This was tipped out when *B. coli* was inoculated.

required by the hog-cholera bacillus to produce a translucency of milk when used as a culture medium. This translucency proceeds parallel with the increasing alkalinity of the medium. The different behavior of the paratyphoid and the hog-cholera bacilli is thus quantitative rather than qualitative, but the difference is such that at a certain time the phenomenon may be used as a qualitative test.

The gradual development of inhibition by the hog-cholera group is paralleled by a gradual loss of inhibition by the paratyphoid bacillus. This was demonstrated in the following manner. A culture of the paratyphoid bacillus, Calf 297, grown in lactose bouillon in a

centrifuge bottle was transferred to fermentation tubes after 7, 24, and 32 days and then inoculated with *Bacillus coli*, Calf 302. The 7 day culture inhibited gas formation completely, the 24 day culture developed 44 per cent gas, the 32 day culture 35 per cent. The three tubes developed the usual amount of acid. In another experiment a culture of paratyphoid bacilli 22 days old yielded after inoculating with *Bacillus coli* 13 per cent gas. It is probable that the inhibition produced after 18 days by the hog-cholera bacillus is gradually lost later on, but no experiments have been made to test this assumption.

The Effect of Killing Paratyphoid Bacilli by Heat on Inhibition of Gas Production by Bacillus coli.

The results of the various experiments made thus far led to a study of the behavior of dead bacilli on gas production. Numerous experiments were carried out with a variety of controls in each but the results were not entirely concordant and pointed to some neglected factor. In all cases the exposure to temperatures which failed to kill the first culture failed to destroy inhibition. After the thermal death point had been reached the results became irregular, but the experiments all agreed in that inhibition was destroyed as the temperature rose and at 100°C. and above gas production was more or less completely restored. A careful analysis of the details of the experiments which are not reproduced here led to the hypothesis that mere death of the first culture is not sufficient to destroy inhibition but that there is another factor involved which disappears rapidly on exposure to high temperatures or gradually at lower incubator temperatures. To demonstrate the gradual disappearance of inhibition the experiments given in Table VII were made. Cultures of paratyphoid bacilli in lactose bouillon contained in large centrifuge bottles were exposed to 62°C. for 35 minutes. Subcultures were made at once and after 1 or more days of incubation to determine whether any bacteria had survived. The culture fluid was transferred to fermentation tubes at once and after the heated fluid had been incubated for 1 or more days. Subcultures were made at each transfer to determine sterility.

TABLE VII.

Effect of Incubation of 5 Day Cultures of Paratyphoid Bacilli (Calf 297) Heated at 62°C. for 35 Minutes on B. coli (Calf 302).

No. of days in incubator.	Gas produced.	Acid in open arm.	Remarks.
	per cent		
0	6–7	4.87	Subculture sterile.
1	5	5.60	" "
3	60	5.10	" "
5	59	5.19	" "
0	4	4.97	" "
4	54	6.00	" "
7	56	5.18	" "

DISCUSSION AND SUMMARY.

Bacteria of the paratyphoid group may be divided into two classes according to the behavior of 4 day cultures in lactose bouillon after a second inoculation with certain types of *Bacillus coli*. One class includes all true hog-cholera bacilli, the other nearly all true paratyphoid and *enteriditis* types. Under the imposed conditions *Bacillus coli* produces the usual amount of gas in the presence of the first group. In the presence of the second no gas or only a bubble appears. The production of acid is not interfered with.

The significance of the inhibition was investigated in a variety of ways suggested by the particular hypothesis entertained at the time. Two main possibilities presented themselves; first, the direct association of the inhibition with living paratyphoid bacilli, and, second, the existence of a ferment or other product of growth as the inhibiting agent.

The theory that the living bacilli or those killed at the lowest possible temperature are responsible was favored by a number of experiments. Thus the complete removal of bacteria by filtration, or by centrifugation combined with the use of kaolin to produce a clear fluid restored gas production. The presence of a fine cloud of bacteria was sufficient to inhibit. On the other hand, the addition of large numbers of living bacteria from agar slants or from lactose bouillon after the requisite incubation period to fresh lactose bouillon

failed to inhibit gas production when *Bacillus coli* was added simultaneously.

When the inhibiting culture was heated at 62°C. for 35 minutes to sterilize it, gas production was still largely inhibited. But it was restored when higher temperatures were used, completely at 100°C. and above. It was also gradually restored by exposing the heated culture to 37°C. for 3 or more days.

The presence of variable amounts of lactose, or even the complete absence of lactose did not interfere with the development of the inhibitory factor.

The activity of the inhibition factor presents itself in the form of a curve, beginning at 0 when both paratyphoid and colon bacilli are inoculated simultaneously and rising as *Bacillus coli* is inoculated at longer intervals from the paratyphoid bacilli. The maximum of inhibition is reached at about the 4th day; thereafter it remains at the same level for a few days and then gradually falls until it is lost within 3 or 4 weeks. The curve of the hog-cholera group is delayed in that the maximum inhibition is reached at the end of 3 weeks. These curves have not been accurately determined. Taking into consideration all the accumulated data the writers tentatively present the hypothesis that the inhibitory factor is some metabolic product of the paratyphoid bacillus, possibly an enzyme, which is destroyed at a temperature somewhat above the thermal death point of the bacilli and which more gradually disappears from incubated cultures. The substance fails to pass Berkefeld filters. It is carried down mechanically with substances clearing the culture fluid.

The experiments support current theories which hold that the acid-producing and gas-producing entities in cultures are distinct.

COMPARATIVE STUDIES ON RESPIRATION.

XII. A Comparison of the Production of Carbon Dioxide by Penicillium and by a Solution of Dextrose and Hydrogen Peroxide.

By F. G. GUSTAFSON.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, June 11, 1920.)

The writer has shown[1] that the production of CO_2 by *Penicillium chrysogenum* is increased by acid and decreased by alkalies. The next step is to investigate the mechanism by which CO_2 is produced. For this purpose it seems desirable to try to construct an artificial system which would imitate the action of the fungus.

In the experiments with *Penicillium* the oxidizable substance was for the greater part dextrose, as the fungus was kept in a 0.5 per cent solution of that substance. A 0.5 per cent dextrose solution was therefore chosen as the basis of the artificial system. From a number of available oxidizing agents hydrogen peroxide was chosen as being the most promising. Dakin[2] states that of the various oxidizing agents hydrogen peroxide comes nearest to bringing about the same reactions *in vitro* as normally take place within the organism. An artificial system was accordingly made which consisted of a 0.5 per cent solution of dextrose plus hydrogen peroxide.[3]

The experiments were performed in essentially the same manner as those already described[1] except that the artificial system was substituted for the fungus. In each experiment the hydrogen ion concentration was kept constant during the whole experiment and the rate of production of CO_2 was measured before and after the addition

[1] Gustafson, F. G., *J. Gen. Physiol.*, 1919–20, ii, 617.

[2] Dakin, H. D., Oxidations and reductions in the animal body, London, 1912.

[3] In these experiments "Dioxygen" was used, as it is nearly neutral and it is stated by the makers to contain only 0.04 per cent of substances other than water and hydrogen peroxide, no preservative being added.

of hydrogen peroxide, while in the experiments with the fungus the hydrogen ion concentration was varied during the experiment. The results are nevertheless comparable in the two cases.

In the final experiments the same bottle of "Dioxygen" was used throughout, so that the results would be exactly comparable. It was found that the contents of two bottles which titrated the same with permanganate did not give the same results in respect to the amount of CO_2 produced. For this reason all the data here published were obtained by the use of hydrogen peroxide from the same bottle.

The time required to cause a standard change in the indicator tube, when the other tube contained 0.5 per cent dextrose but no hydrogen peroxide, was taken as 100 per cent. This change may have been caused by a leak in the apparatus or by the breaking down of the dextrose or by both. The source of the CO_2 is of no importance for the measurement. It is only necessary that in the absence of hydrogen peroxide the rate of production of CO_2 should be constant so that the rate found after the introduction of the hydrogen peroxide could be compared with it. For convenience the rate in the absence of hydrogen peroxide is called the normal rate; the rate after the addition of hydrogen peroxide is expressed in terms of the normal. This normal change was very slow and varied between 20 and 35 minutes.

In beginning an experiment the 0.5 per cent dextrose solution was made up to the desired hydrogen ion concentration; 60 cc. of this solution were put into the apparatus and the air was caused to circulate for 2 hours or more till all dissolved CO_2 had been pumped out. Several readings were then taken to get the normal rate of change. Then 8 cc. of hydrogen peroxide were added by opening the flask holding the dextrose solution; 3 minutes were allowed to elapse before the first reading was taken so as to allow a thorough mixing of the solution. In the first few minutes the production of CO_2 was very great, but as will be seen from Fig. 1, it fell off very rapidly in the solution having a pH of 1, while in the neutral solution the fall was not so rapid.

The experiments with the alkaline solutions were made with the direct method (without the apparatus for the circulation of air) as described in a previous paper.[1] Experiments were first made with neutral solutions. The dextrose solution was placed in a tube,

hydrogen peroxide was then added, and 4 minutes were allowed to elapse. The time required to produce a definite[4] amount of CO_2 was then measured. The time it took to produce the same amount of CO_2 in the alkaline solution was then compared with the time in the neutral solution.[5] In Fig. 1, the comparison is expressed by taking

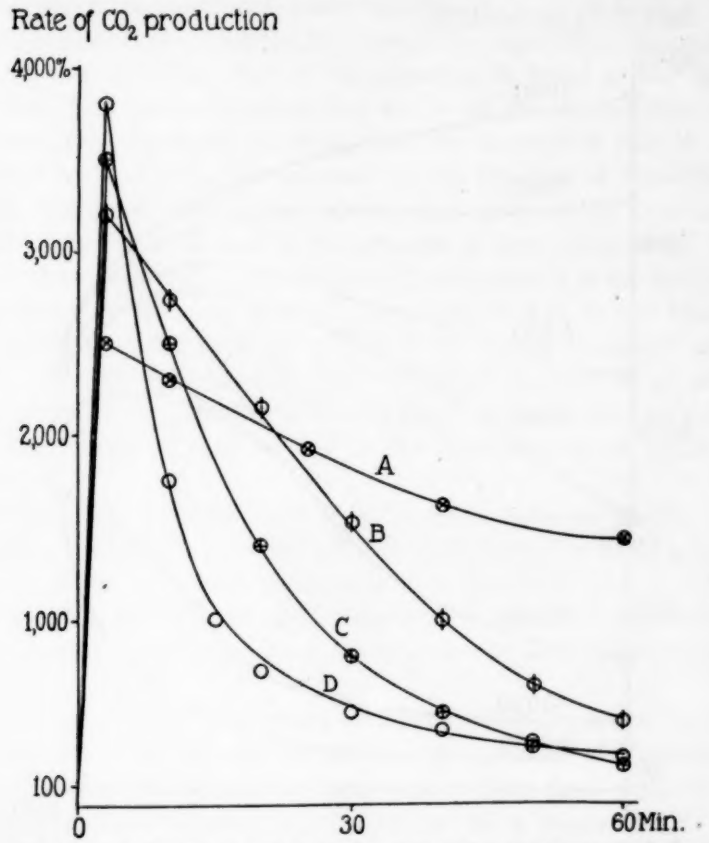

FIG. 1. Curves showing the rate of production of CO_2 by a solution of dextrose and hydrogen peroxide. Curve A shows the rate in a solution having a pH of 8.75, B in a solution of pH 7, C in a solution of pH 5, D in a solution of pH 1. The rate in the absence of hydrogen peroxide is taken as 100 per cent.

[4] This was ascertained by titrating with CO_2 as described in a former paper. (Gustafson, F. G., *J. Gen. Physiol.*, 1919–20, ii, 617.)

[5] The experiments with alkaline and neutral solutions were carried out almost simultaneously.

for the alkali curve a proportional part of the ordinate of the neutral curve obtained by the use of the apparatus. Thus if by the direct method the rate of production of CO_2 in the alkaline solution was two-thirds as great as in the neutral solution it was assumed that this would also be the case if the experiment could be performed in

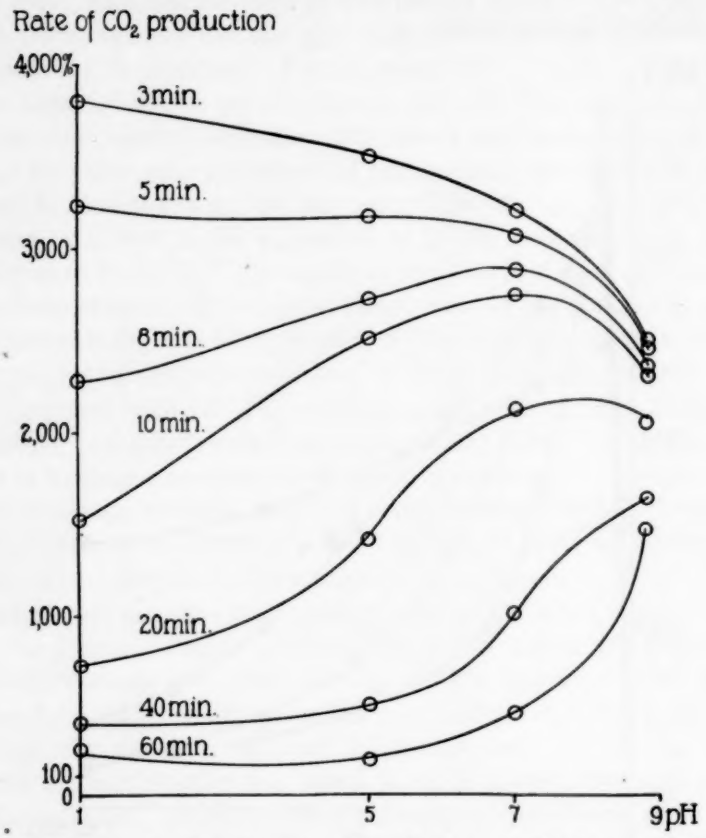

FIG. 2. Curves showing the rate of production of CO_2 by a solution of dextrose and hydrogen peroxide as related to the acidity of the solution.

the apparatus and accordingly the ordinate of the alkali curve in Fig. 1 was made equal to two-thirds the ordinate of the neutral curve.

From these experiments it was found that the production of CO_2 in the alkaline solution was at first below that at neutrality, but the fall in production was less rapid, and as will be seen from Figs. 1 and

2 the rate was higher at the end of an hour in the alkaline solution than in the neutral solution. The decrease in production of CO_2 in the alkaline solution is not so great as that shown by the curve, because in taking more than one reading the solution had to be made alkaline again by adding more NaOH, and this produced a buffer action, which increased with every reading.

It is evident that during the first part of the experiment the production of CO_2 resembles that of the organism in being greater in an acid than in a neutral medium, but less in an alkaline medium than at neutrality. It should be noted that the increase of rate in acid followed by a decrease is also observed in the case of *Penicillium*.

The falling off of the rate in the latter part of the experiment might be due to a decrease in the amount of some substance. Evidently this is not the sugar; it is doubtful whether it is the hydrogen peroxide, since titration with permanganate as well as the catalase test shows that the hydrogen peroxide has hardly decreased at all by the time the rate has fallen off. It might possibly be an active form of hydrogen peroxide or a catalyzer. It is of course possible that the falling off may be due to the formation of an inhibitory substance.

According to Spoehr[6] there is no reaction between glucose and hydrogen peroxide in a neutral solution, but in a 0.5 M KOH solution 2 gm. of glucose had been completely used up at the end of 3 days, forming CO_2, formic acid, glycocollic acid, and α-hydroxymethyl-*d*-arabonic acid. As far as the writer is aware no work has been done with acid solutions.

It is probable that the reason why Spoehr did not notice any production of CO_2 in the neutral solution was because the amount is very small and the production lasts only a short time, while in the alkaline solutions the production continues for a longer time.

SUMMARY.

A neutral solution of dextrose and hydrogen peroxide acts like *Penicillium chrysogenum* in producing an increased amount of CO_2 upon the addition of acid, but not upon the addition of alkali.

[6] Spoehr, H. A., *Am. Chem. J.*, 1910, xliii, 227.

DYNAMICS OF NERVE CELLS.

I. The Temperature Coefficient of the Neurogenic Rhythm of the Heart of Limulus polyphemus.

By WALTER E. GARREY.

(*From the Physiological Laboratory of the Tulane University Medical School, New Orleans.*)

(Received for publication, June 1, 1920.)

The temperature coefficient of the rate of heart beat has been determined by many investigators on various animals both vertebrate and invertebrate.[1] In all it was found that the coefficient for 10°C. was approximately 2 in the intermediate ranges of temperature, greater at lower temperatures, and somewhat less at high temperatures.

In none of these determinations has it been possible, owing to the character of the hearts worked with, to determine whether the results were due to an effect of temperature on the coefficient of ganglionic activity, of muscular activity, or of both.

It has therefore seemed of importance to determine whether variations of temperature, affecting ganglion cells alone, would give temperature coefficients of the same order of magnitude as those obtained on the whole heart.

A suitable preparation for this purpose is the excised heart of *Limulus polyphemus*, the beats of which have been shown by Carlson to be purely neurogenic.[2] Carlson has also described methods by which the ganglion can be separated from the muscle and independently subjected to variations of temperature while the muscle records the beats.

[1] For a general review of the literature of this subject see Kanitz, A., Temperatur und Lebensvorgänge, Berlin, 1915. Loeb, J., The Organism as a whole, from a physicochemical viewpoint, New York, 1916. Snyder, C. D., *Am. J. Physiol.*, 1911, xxviii, 167.

[2] Carlson, A. J., *Am. J. Physiol.*, 1905–06, xv, 220; also *Ergebn. Physiol.*, 1909, viii, 427.

The ganglion of the heart of *Limulus polyphemus* occupies a median dorsal position on that organ; the nerve cells occupy a position over the posterior five or six segments of the heart and are connected with the anterior muscular segments by a median and two lateral nerves. This ganglion, which may be 10 or 15 cm. in length, can be dissected from the underlying tissue with ease and maintain functional connection with the muscle of the anterior segments.

In the experiments to be described a method was finally adopted in which a Dewar flask contained an immersion fluid, either sea water or *Limulus* blood serum. A system of glass tubes provided for renewal of the fluid at different temperatures which were recorded by a standard thermometer graduated to 0.1°C. Either the posterior segments of the whole heart, or the dissected ganglion, depended into the fluid through an opening in a paraffined cap. The two anterior segments were pinned to the cap, enclosed in a moist chamber, and kept at a constant temperature. The contractions of the anterior segments were graphically recorded and served as an index not only of the rate but of the effective strength of the impulses reaching the muscles. The study in its ramifications has extended to about 200 *Limulus* hearts.

Observations on the Whole Heart.

At Beaufort, N. C., the laboratory sea water was 27.7°C. and the average rate of 60 hearts was 23 beats per minute, while, at Woods Hole, the laboratory sea water was 20.1°C. and the average rate of 48 hearts was 11.6 per minute. A temperature coefficient of about 2.6 (Q_{10}) is obtained from these figures, calculating for the 10° interval. The individual hearts show wide variations in rate, and since the sea water of the two localities differs somewhat in concentration, this average coefficient for the whole heart can be considered only an approximation.

To illustrate the magnitude of the temperature coefficient of the whole heart for what may be considered a fairly normal range of temperature, ten experiments have been selected from our series—experiments in which it so happened that observations were made at exactly 15 and 25°C. The results are given in Table I. The experiments given in Table I show that the whole heart of *Limulus*, subjected to

15 and 25°C. respectively, has rates giving an average temperature coefficient (Q_{10}) equal to 2.23. This indicates that the rate is determined by the alteration of a chemical process. The coefficient is quite in conformity with that reported for other rhythmic biological processes for this range of temperature.

TABLE I.

Rate per min. at		Temperature coefficient. (Q_{10})
15°C.	25°C.	
6	15	2.5
6.1	12	1.9
9	23	2.4
7.5	14.2	1.8
8	19	2.3
8.5	17	2.0
5	12	2.4
6.8	13.5	2.0
7.2	20	2.7
7	16	2.3
Average...7.1	16.17	2.23

In order to obtain a more comprehensive conception of the variation in the temperature coefficient of the whole heart subjected to different temperatures, summaries of some experiments are given below. The first shows the effect of cooling the heart to −2°C. and then progressively warming in a single but typical experiment.

Temperature range.	Temperature coefficient. (Q_{10})	Temperature range.	Temperature coefficient. (Q_{10})
°C.		°C.	
8 to −2	3.7	25 to 15	2.8
10 " 0	3.3	28 " 18	2.5
13 " 3	3.2	30 " 20	2.2
15 " 5	3.1	33 " 23	1.9
18 " 8	3.2	35 " 25	1.7
20 " 10	3.0	38 " 28	1.5
23 " 13	2.9		

The progressive change from a large coefficient at low temperatures to the smaller values at high temperatures is not always as uniform

as illustrated in this experiment, but the coefficients at very low temperatures are usually larger than those recorded above.

Extremes of temperature may alter the value of temperature coefficients subsequently determined. It seems best in view of this fact to show separately the effects of depression and of elevation of the temperature starting with the normal temperature of the sea water. Each series thus is based on observations only on one side of the normal temperature, *i.e.* between it and a single limiting temperature, and thus the secondary effects of excessive heat or cold are avoided. In Table II the results of ten experiments are given in each of which the posterior seven segments of the whole heart with its ganglion were subjected to temperature variations.

TABLE II.

Temperature range.	Temperature coefficient (Q_{10}).						Temperature range.	Temperature coefficient (Q_{10}).			
	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.	Experiment 6.		Experiment 7.	Experiment 8.	Experiment 9.	Experiment 10.
°C.							°C.				
−2 to 8	6.0	4.6			7.6	7.2	20 to 30	2.3	2.5	1.8	
0 " 10	3.5	3.9	4.1	3.7	5.4	5.0	21 " 31	2.3	2.2		2.0
3 " 13		3.6		2.7			23 " 33		1.9	1.9	2.1
5 " 15	2.8	3.1	3.3	2.2	2.8	3.2	25 " 35	1.8	1.7		1.92
8 " 18		3.2		2.3	2.6		27 " 37				
10 " 20	2.5	2.8	3.0	2.0	2.66	3.0	28 " 38	1.6	1.5	1.7	1.67
11 " 21					2.5						

Observations on the Ganglion.

We may now compare the above results with those obtained by varying the temperature of the ganglion alone after dissecting it free from all adherent muscle tissue except the anterior segment. It would seem best for purposes of comparison with the results obtained above on the whole heart to observe the same precautions and to avoid the effects due to exposure of the ganglion to excessive heat or cold. The results tabulated in Tables III and IV are thus strictly comparable to those given in Table II since they show the simple effect of a progressive change in the temperature of the ganglion above or below the initial normal temperature of 20°C. In order to illus-

TABLE III.

Effects of Cooling the Heart Ganglion Alone.

Experiment.	Temperature.	Rate per min.	Coefficient for 10°C.	
			Temperature range.	(Q_{10})
	°C.		°C.	
1	20	17	20 to 10	2.2
	17	13	17 " 7	2.9
	15	11.7	15 " 5	2.9
	10	6.2	10 " 0	4.3
	7	4.4	7 " −2	4.5
	5	4	5 " −2	7.2
	0	1.4		
	−2	1		
2	20	8.6	20 to 10	1.9
	15	6.4	15 " 10	2.1
	10	4.4	15 " 5	2.1
	5	3	10 " 0	2.9
	0	1.5	5 " 0	4.0
3	20	16	20 to 10	2.5
	15	10.5	15 " 5	2.8
	10	6.2	10 " 0	3.5
	8	5	8 "−2	6.0
	5	3.7	5 "−2	8.9
	0	1.7		
	−2	0.8		
4	21.6	11.8	21.6 to 11.6	2.8
	16.5	7.0	16.5 " 6.5	3.3
	11.6	4.2	11.6 " 1.6	5.6
	6.5	2.3		
	1.6	0.74		
5	19.7	12.6	19.7 to 10	2.3
	16	9	16 " 6	2.1
	12	6.6	12 " 2	2.2
	10	5.4	10 " 0	2.4
	6	4.3		
	2	3		
	0	2.2		

trate the widest range of variation in experimental results, experiments on eight different animals were selected arbitrarily. As was expected the cardiac rate was slow when the ganglion was cooled and fast when it was warmed. In the lower ranges of temperature the temperature coefficient (Q_{10}) is well above 2 and at very low tempera-

TABLE IV.

Effects of Warming the Heart Ganglion Alone.

Experiment.	Temperature.	Rate per min.	Coefficient for 10°C.	
			Temperature range.	(Q_{10})
	°C.		°C.	
6	20	8	20 to 30	2.5
	22.5	12	22.5 " 32.5	2.1
	25	14	25 " 35	2.0
	28	20	28 " 38	1.6
	30	23		
	32.5	26		
	35	28		
	38	32		
7	20	12	25 to 30	2.2
	22	15	22 " 32	2.0
	25	17.5	25 " 35	1.9
	27	20	27 " 37	2.1
	30	27		
	32	31		
	35	33		
	37	35		
8	21	18.3	21 to 26	2.0
	26	26.8	21 " 31	1.8
	31	33	26 " 36	1.5
	36	40		

tures may be as large as 9. This places the process upon which the rate of the rhythmic ganglionic discharge depends unequivocally within the class of chemical reactions. The decrease in the coefficient at higher temperatures is the rule in both biological and chemical reactions and does not militate against this interpretation. The results in Tables III and IV are illustrated in Fig. 1.

If we now compare the results obtained on the whole heart with those obtained when the ganglion alone is subjected to changes of temperature we are struck with the fact that there is not a single feature of the temperature coefficients which will serve to differen-

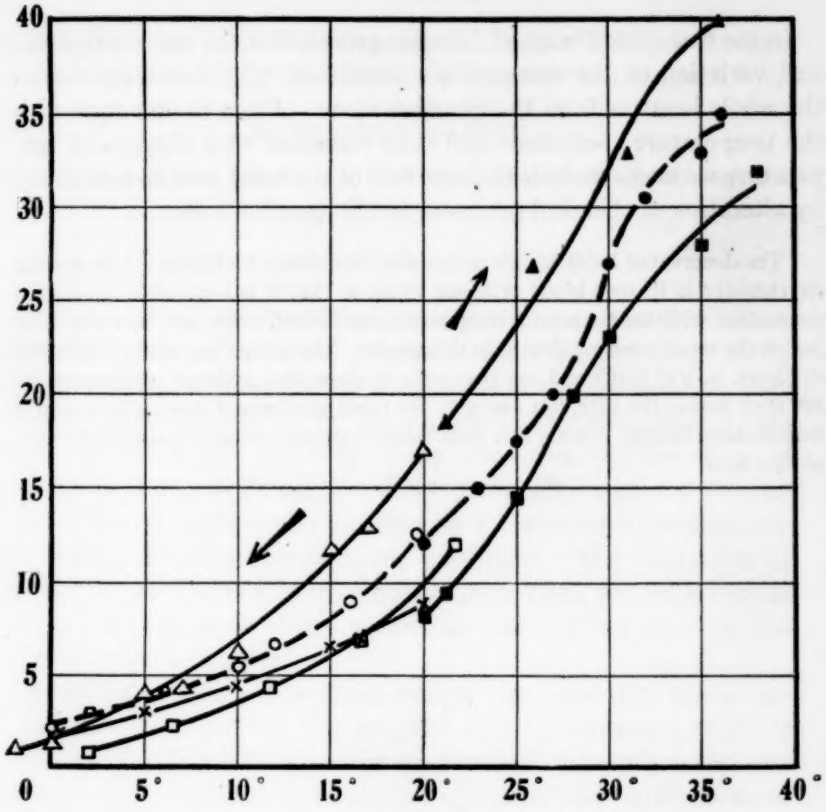

FIG. 1. Curves for the rates plotted against temperature. Data from Tables III and IV. △, ○, ×, and □ correspond to 1, 5, 2, and 4 respectively and show the effects of progressive cooling; ▲, ●, and ■ correspond to 8, 7, and 6 respectively.

tiate one from the other. The magnitude of the temperature coefficient and its variations are parallel in the two cases and we are justified in concluding that there is the same typical temperature coefficient for the rate of the heart beat of *Limulus* when the ganglion alone is

subject to variations of temperature as when the whole heart is similarly treated. Furthermore chemical alteration in the ganglion determines the rate of heart beat.[3]

CONCLUSIONS.

In the case of the heart of *Limulus polyphemus* the same magnitude and variation of the temperature coefficient (Q_{10}) is obtained from the whole heart as from the ganglion alone. From the magnitude of the temperature coefficients and their variation with changes of temperature we may conclude that the rate of the heart beat is determined by alteration of chemical processes in the ganglion cells.

[3] The denervated heart muscle of *Limulus* was shown by Carlson to be without rhythmicity in its own blood or in sea water so that it is impossible to speak in connection with the tissue of a temperature coefficient which can have any bearing on the results communicated in this paper. The author has made this muscle rhythmic in M/2 NaCl and has been able to show that a rise of temperature of 10° may double the rate, but owing to the rapid incidence of toxic effects and to concomitant changes in rate, it is impossible to present reliable quantitative data at this time.

DYNAMICS OF NERVE CELLS.

II. The Temperature Coefficients of Carbon Dioxide Production by the Heart Ganglion of Limulus polyphemus.*

By WALTER E. GARREY.

(From the Physiological Laboratory of the Tulane University Medical School, New Orleans.)

(Received for publication, July 24, 1920.)

That the rate of the heart beat of *Limulus polyphemus* is determined by the rate of chemical reactions within the nerve cells of the cardiac ganglion is shown by the magnitude of the temperature coefficient (Q_{10}) as determined by the author.[1] The differences in the temperature coefficient, the form of the rate curve, and the effects of extremes of temperature suggested the probability that the velocity of the chemical reaction was modified by a consecutive physical process with a different temperature coefficient. The formation of CO_2 in the nerve cells and its diffusion from them are suggested as processes analogous to those underlying the determination of the rate of impulse formation.

The following report shows that changes in temperature affect carbon dioxide formation by the ganglion cells in a manner which is quantitatively similar to the effect on the rate of the rhythmic contractions, thus establishing a basis for a hypothetical explanation to account for the different magnitude of the temperature coefficient at different ranges of temperature, and also for the change in the rate curve as affected by extremes of heat and cold.

* The experimental work was done at the Marine Biological Laboratory, Woods Hole, Massachusetts. The author wishes to express his appreciation of the courtesy of the Director and staff of the Laboratory for placing the facilities for this work at his disposal.

[1] Garrey, W. E., *J. Gen. Physiol.*, 1920–21, iii, 41.

Method.

The rate of formation of even the small amount of carbon dioxide evolved from ganglia, each weighing only 12 to 30 mg., is not difficult if one uses the indicator method of Sörensen[2] as modified for tissue work by Osterhout and his collaborators.[3] The method of Tashiro,[4] who demonstrated that the *Limulus* ganglion produces CO_2, did not admit of rapid variation of the temperature and was not adaptable to our purpose.

The indicator method was used with the technique and precautions described by Moore.[5] The velocity of the reaction at different temperatures is determined by finding the time required to change the hydrogen ion concentration of an immersion fluid from pH 7.7 to 7.4 using phenolsulfonephthalein as the indicator.[6]

Buffer must be avoided in the immersion fluid which was a balanced saline solution of the following composition: 100 parts of M/2 NaCl, 1.5 parts of M/2 KCl, and 2.2 parts of M/2 $CaCl_2$, to 1 liter of which 15 cc. of 0.01 per cent phenolsulfonephthalein were added. The desired initial alkalinity (pH 7.7) was obtained by adding the requisite amount of NaOH.

The color comparisons were made in "Pyrex" glass tintometer tubes of about 4 cc. capacity, all of the same diameter, and filled with solution to a 3 cc. graduation mark. The tubes were stoppered with corks impregnated before each experiment with neutral, boiling paraffin or with rubber stoppers boiled in distilled water, paraffin-coated, and assuredly free from acid. The tubes and fluid were brought to the desired temperature before the ganglion was introduced and then kept at a constant temperature in large vessels of water. At the low temperatures it sometimes became necessary to adjust the tempera-

[2] Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131; *Ergebn. Physiol.*, 1912, xii, 393.

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17, 171. Gustafson, F. G., *J. Gen. Physiol.*, 1918–19, i, 181. Brooks, M. M., *J. Gen. Physiol.*, 1918–19, i, 193. Thomas, H. S., *J. Gen. Physiol.*, 1918–19, i, 203. Irwin, M., *J. Gen. Physiol.*, 1918–19, i, 209.

[4] Tashiro, S., A chemical sign of life, Chicago, 1917.

[5] Moore, A. R., *J. Gen. Physiol.*, 1918–19, i, 613.

[6] The author is indebted to Prof. A. R. Moore who cooperated in the initial experiments of this investigation.

ture by the addition of cold water or ice to the large containers but the variations in temperature were slight and negligible.

With scrupulous care to avoid injury, the ganglion was dissected from the heart and freed of all adventitious tissue, rapidly weighed, and immersed in the fluid (pH 7.7) to wash away all possible trace of acid and buffer. It was then placed in the tintometer tube at the desired temperature and the time required to assume the tint (pH 7.4) was determined with a stop-watch.

Results.

The results obtained in five experiments are presented in Table I. The relative velocity of CO_2 production is the reciprocal of the time

TABLE I.

Rate of Carbon Dioxide Production by Ganglia.

Temperature.	A Weight = 18 mg.		B Weight = 22 mg.		C Weight = 26 mg.		D Weight = 25 mg.		E Weight = 16 mg.	
	Time.	Q_{10}	Time.	Q_{10}	Time.	Q_{10}	Time.	Q_{10}	Time.	Q_{10}
°C.	*sec.*		*sec.*		*sec.*		*sec.*		*sec.*	
24	214		165		115		135		68	
14	393	1.8	345	2.1	319	1.9	445	3.3	282	4.0
4	1,800	4.5	1,245	3.6	778	2.4	1,920	4.3	1,480	5.2
14	247	7.4	290	4.3	290	2.6	645	3.0	360	4.1
24	75	3.3	111	2.6	144	2.0	180	3.5	108	3.3
34	37	2.0	65	1.7			110	1.6	59	1.8

in seconds required to change the tint of 3 cc. of solution from pH 7.7 to 7.4. The figures given are averages, usually of three readings, which in the present instance did not vary more than 3 per cent from the mean.

The results shown in Experiments A, B, and C (Table I) are typical. The temperature coefficient (Q_{10}) is equal to or greater than 2, in all except the highest range of temperature. The magnitude of the coefficient is of the same order as that previously found by Garrey[1] for the rate of the heart beat. Another similarity to the effects of temperature on rate of rhythm is to be found in the fact that it is usual that transient exposure to temperatures as low as 4°C. so affects

the nerve cells that, after the temperature is again raised, the activities are pitched at a higher level than that maintained at this temperature prior to cooling. But while this is usual it is not an invariable result of cooling as is shown by the two experiments summarized in D and E (Table I).

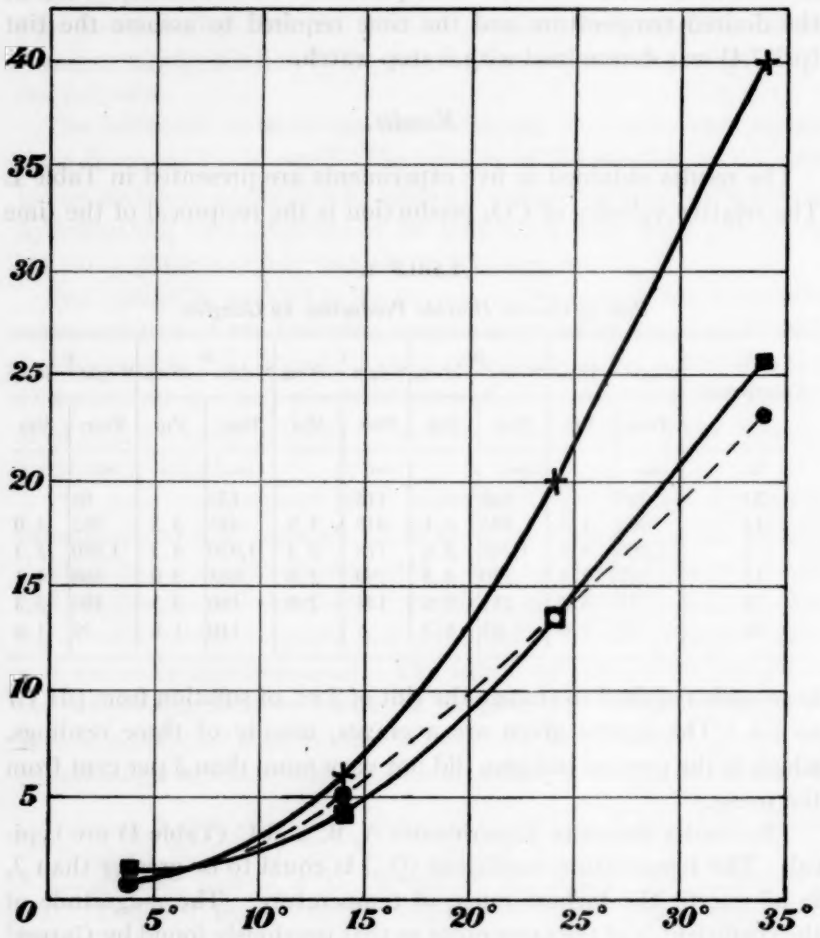

FIG. 1. Curves of the relative velocities of CO_2 production by *Limulus* heart ganglion at different temperatures. The data from Experiments A, B, and E, Table I. The reciprocal of time in seconds is multiplied by 1,500.

The relative velocities with which the nerve cells form CO_2 at different temperatures are also shown graphically in Fig. 1. The curves are obtained from data in Experiments A, B, and E of Table I and are constructed by plotting the reciprocal of time in seconds multiplied by a constant (1,500). These curves of chemical reaction velocities, like those of rate of heart beat, are exponential and not linear functions of the temperature.

Certain exceptions to the rule that the temperature coefficients of heart rate were large at low temperatures and decreased with a rise of temperature have been reported by the author. We submit below data which indicate an exception which likewise applies to the formation of CO_2 by the ganglion. The ganglion in this case was the largest obtainable weighing 32 mg. from a *Limulus* weighing 8½ pounds.

Temperature.	Tint matched after.	Q_{10}
°C.	*sec.*	
27	155	
37	72	2.1
27	175	2.4
37	79	2.2
27	160	2.0
37	76	2.1
27	153	2.0
17	300	2.0
27	153	2.0

The above data show the usual temperature coefficient (Q_{10}) equal to about 2 for the range 17–27°C. but the coefficient for the upper range, 27–37°C., is uniformly larger than that usually found in our other experiments. It is also seen that after the first treatment of the ganglion at 37°C. and then cooling to 27°, the rate of CO_2 development is somewhat slower than at 27° prior to the heating. This is usual but it should also be noted that at the end of the experiment the rate at which the carbon dioxide was developed was identical to the initial rate at 27°C. It is evident from this result that even frequent subjection of the ganglion to 37° did not cause injury but produced effects which were entirely reversible in character. The constancy of the coefficients (Q_{10}) at different temperature levels in

this case is suggestive of the results found by Loeb and Wasteneys[7] for oxidation processes in *Arbacia* eggs between 3 and 30°C.

It might be supposed from the results recorded in the preceding pages that CO_2 production is not proportional to the ganglionic mass since the rate at which carbon dioxide is produced is often very different for ganglia of equal weight, but it is also true that the rates at which hearts of equal size beat likewise show normally such variations. These facts merely mean that the rate of metabolic activity of different ganglia is different, for if we make the comparison with tissue from a single ganglion we find that the CO_2 development is proportional to the mass. In the following experiment showing this, the whole ganglion weighed 21 mg. Its rate of CO_2 development

TABLE II.

Relation of Ganglionic Mass to Rate of CO_2 Production.

Temperature.	Whole ganglion = 21 mg.		Anterior part = 10 mg.		Posterior part = 11 mg.	
	Time.	Q_{10}	Time.	Q_{10}	Time.	Q_{10}
°C.	*sec.*		*sec.*		*sec.*	
24	75		145		140	
14	160	2.1	340	2.3	325	2.3
4	510	3.1	1,200	3.5	1,095	3.3
14	150	3.4	330	3.6	275	4.0
24	60	2.5	150	2.2	120	2.3
34	35	1.8				

at different temperatures was determined and the ganglion then divided so that the posterior portion weighed 10 mg. and the anterior part 11 mg. After division, the parts were kept for a short time in neutral saline solution to avoid any complication from the acid which might have developed due to the injury; the rate of CO_2 development by each portion was then determined for the temperatures to which the whole ganglion had previously been subjected. The results are given in Table II and show conclusively that in a given ganglion the carbon dioxide production is proportional to the mass of ganglionic tissue.

[7] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxvi, 345.

That the cardiac ganglia of different *Limuli* should show individual differences in the rate of carbon dioxide development is not surprising but the physiological significance of the metabolic variations is not so apparent. In our first paper[1] attention was directed to the fact that there were marked individual variations in the rate of the cardiac rhythm of different *Limuli* but that all showed temperature coefficients indicating the chemical character of the causative reaction. The present investigation was undertaken to determine whether carbon dioxide development was affected by temperature like the heart rate, and while it has been demonstrated that this is the case it has yet to be shown that, given two ganglia of equal weight but with different heart rates, the rate of carbon dioxide development is proportional

TABLE III.

Relation of Cardiac Rate to Development of CO_2.

Temperature.	A Weight of ganglion = 18.4 mg.		B Weight of ganglion = 17.7 mg.	
	Heart rate per min.	CO_2 development. Time.	Heart rate per min.	CO_2 development. Time.
°C.		*sec.*		*sec.*
22	16	105	8	185
12	7	240	3	495
Q_{10}.....	2.3	2.3	2.6	2.7

to the rate of the rhythm. To settle this point two *Limuli* of the same size were selected which showed heart rates of 8 and 16 per minute respectively at room temperature (22°C.). The hearts were excised and the rate of each at 12° was determined, after which the ganglia were dissected from the hearts and the rate of carbon dioxide development was then determined. The results, compared with the previous rates, are given in Table III.

The results of this experiment (Table III) leave no doubt that the rate of CO_2 development by the ganglia and the rate of cardiac rhythm run parallel courses.

All the experimental results herein reported thus show that the temperature coefficients of carbon dioxide production by the ganglia are of a magnitude entirely commensurate with those previously re-

ported by Garrey for the rate of neurogenic rhythm of *Limulus* hearts. The data strongly support the view that the chemical process within the nerve cells which is responsible for the rate of the heart beat is one which is associated with the development and rate of diffusion of carbon dioxide.

SUMMARY.

1. It is possible to determine by the colorimetric method the rate of production of carbon dioxide by the cardiac ganglion of *Limulus*.

2. Carbon dioxide formation in the cardiac ganglion was found to run parallel to the rate of heart beat for different temperatures.

3. The conclusion seems justified that the rate of cardiac rhythm of *Limulus* depends upon a chemical reaction in the nerve cells of the cardiac ganglion and that this reaction is associated with the production of carbon dioxide since the rate of beat and the rate of CO_2 production are similarly affected by changes in temperature.

ON THE RÔLE OF AN INTEGUMENTARY PIGMENT IN PHOTORECEPTION IN HOLOTHURIA.

By W. J. CROZIER.

(Contributions from the Bermuda Biological Station for Research, No. 117, and from the Hull Zoological Laboratory, University of Chicago, Chicago.)

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The experiment recorded in this note was undertaken in 1917, with a view to testing the notion (Crozier, 1914–15) that the fluorescent, greenish yellow, integumentary pigment characteristic of some members of the genus *Holothuria* is concerned with their photic sensitivity. A functional connection of this sort was suspected because the presence and relative amount of the pigment seemed to be correlated with the degrees of photic stimulation in three species of *Holothuria* studied at Bermuda; a pigment of similar nature seems to occur (Crozier, 1917) in the West Indian *Actinopyga agassizii* which, like these species of *Holothuria*, is negatively heliotropic. Dubois (1914), in his book, had earlier suggested a photosensitizing rôle for the fluorescent (dichromatic) substances occurring in various worms and echinoderms. It was expected that the stimulating power of sunlight transmitted by a screen of this pigment might be different from that of sunlight directly falling upon one of these holothurians. It is known that in photochemical action the effects of light are due to the absorbed wave-lengths. With the skin pigment of *Holothuria* there is pronounced absorption in the visible spectrum. Hence it was assumed that light deprived of these absorbable rays, by preliminary passage through a layer of the pigment, might perhaps be less effective for excitation. This expectation was not realized.

The species employed was *Holothuria captiva*, a form very sensitive to light, which was usually found under stones; in dark caves, however, it was found creeping freely over the walls, showing that its occurrence under stones along the shore is in all probability due to its photic irritability.

The time required for the orientation of individuals 6.0 to 6.5 cm. long by sunlight reflected from a mirror, was found to be 6 to 8 minutes at 20°C.; the animals were at the beginning placed at right angles to the incident light. Other things constant, the speed of orientation varies inversely with the light intensity. The same individuals, and others in addition, were then in a similar manner exposed to sunlight which had passed through an alcoholic solution of the integumentary pigment of this species. A thickness of solution was used sufficient to produce apparently complete absorption of wave-lengths shorter than 530$\mu\mu$; the pigment of *Holothuria captiva* does not exhibit a band spectrum (*cf.* Crozier, 1915).

It was found that under the influence of the filtered sunlight, orientation required on the average 6 minutes—the same time as noted with the unfiltered light.

The photodynamic activity of eosin exhibits a peculiarity, discussed by Huber (1905) and by Clark (1918–19), which corresponds in certain respects with the apparent failure of a screen of *Holothuria* pigment to "protect" the animal to any degree from the stimulating action of light. The photodynamic action of eosin on rennin is found not to be materially lessened by the interposition of an eosin screen; hence the fluorescence of the photodynamic agent cannot be regarded as essentially concerned in any way with the production of the toxic effects. Clark (1918–19) has suggested that in such cases the essential photodynamic action (production of toxic materials) may depend upon wave-lengths for which the fluorescent substance is partially transparent.

Light which has passed through an adequate layer of *Holothuria* pigment will not excite fluorescence in a subsequent layer, hence the fluorescence must be due to wave-lengths readily absorbed. But light filtered in this way is not detectably weakened as a stimulating agent[1] for this animal. It must therefore be presumed that, if the pigment is in reality implicated in photoreception, light not readily absorbed by it is concerned in certain photochemical transformations differing from those which find expression in dichromaticity.

[1] Note that in this experiment the criterion of stimulating power has to do with the *rate of orientation* of the tested organisms—not with the rate of locomotion.

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STUDIES IN THE DYNAMICS OF HISTOGENESIS.

II. Tension of Differential Growth as a Stimulus to Myogenesis in the Esophagus.

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(Received for publication, April 3, 1920.)

INTRODUCTION.

Facts presented, for the first time, in a previous publication by the writer (1919–20) prove that the developing descending colon of the pig embryo possesses two zones of differential growth which by their interaction mutually influence each other during the formation period. The inner epithelial tube is the dominant, most active region of growth. It presents numerous mitotic figures which pursue a path cephalad, primarily, in the manner of a left-handed helix. The outer mesenchymal zone is less active in growth and early in development is composed of a uniform mass of undifferentiated cells.

The inner, rapidly growing, epithelial tube practically revolves due to the rapid spiral growth of its cells. With subsequent growth an apparent ring (this is qualified apparent for in reality a close spiral is formed) of smooth muscle myoblasts appears gradually near the periphery of the vortex.

This position taken by the inner, close spiral, smooth muscle coat at some distance from the epithelial tube is dependent upon an optimum tension. The attitude heretofore taken by embryologists eliminated the search for the underlying cause of the first formed, inner, muscle coat The theory of self-differentiation excluded interpretation as regards myogenesis. A certain mesenchymal cell, regardless of position, was considered as destined to become a myoblast.

This highly differentiated tissue is now considered to self-develop, for so called muscle-forming elements have been identified in the

ovum (Conklin; Wilson). By the exclusion of this element in sectioning experiments the subsequent, positional, environmental relation is as much destroyed as the absence of the myoplasm. It may well be that the yellow pigmented zone is destined to assume a certain subsequent relation in development. This position may necessitate subjection to an optimum tensional stress stimulus due to the differential growth. The conclusion of self-differentiation is consequently unwarranted and too broad. All the work tending to support the generalization that muscle self-differentiates excludes the inner, environmental stimulus—the stretching or tensional stimulus of differential growth. Has the isolated myoplasm been cultured and found to form muscle? Only the affirmative answer to this question will warrant the assertion that muscles self-develop and then only providing the exclusion of surrounding germ plasm has been accomplished so as to exclude totally tensional stresses of differential growth of relational parts.

The last statement is made due to the fact that W. H. Lewis came to the conclusion that muscle self-differentiates from experiments in which transplantation of tissues around the otic capsule of tadpoles was performed. That musculature subsequently appears is not to be wondered at, for the potencies or actualities of differential growth were also misplaced with the transplant. If this piece remained viable it was bound to reveal subsequently the same tissues as in its normal location for the resultants of differential growth and the potential, mechanical stimulus due to space relation were left intact.

Consequently, the potencies of a blastomere are as much a function of its position as of its material substances. The material substances receive and react to the stimulus. The stimulus is a function of position. To elicit the response of mesenchymal cells in the formation of muscle tissue the proper optimum tensional stress stimulus must be applied. In tissue differentiation, therefore, the stimulus as well as the reception and response must be taken into consideration.

Tensional stresses are of various kinds and degrees. The quantity as well as the quality of stretching is important. The connective tissues are resultants of certain degrees of stresses. Muscular tissues, on the other hand, are responses to still different types of stresses.

The submucosa interposed between the epithelial tube and the inner smooth muscle coat presents cells which react to a certain minimum of tensional stress. Just peripheral to the submucosa, muscular tissue is differentiated as a response to an optimum tensional stress.

It was observed by von Uexkull that in the nerve net of invertebrates the excitation flows into a stretched muscle. Extension, stretching, or elongation of a muscle cell precedes the desired effective contraction, therefore, as was inferred long ago by Hunter from observations on mammalian muscular action. It was also found by Cannon that there was a subliminal, an optimum, and a supermaximal tensional stimulus to elicit the response of the contractile tissue of the stomach in the normal stomachic movements. Evidently, an analogy is here found for the development of the musculature. There appears to be a subliminal, an optimum, and a supermaximal tension for stimulating the formation of contractile tissue. In normal development as well as in subsequent normal function the tensional stresses appear to be fundamentally involved.

Tension is due to a definite mechanical action. The formation of muscular tissue is due, therefore, to a definite active process, not a passive one as the term self-differentiation connotes. Loeb likewise concludes from his experiments on the gastrocnemius muscle of the frog that growth is an active, not a passive, affair as follows: "Activity, therefore, plays the same rôle in the growth of a muscle that the temperature plays in the growth of the seed."

The dominant growth of the epithelial tube and the resultant tension or stretching of the surrounding mesenchymal cells is strikingly exemplified throughout the digestive tract in its early stages of development. The object of the first part of this paper, therefore, is to demonstrate this interaction in the esophagus; furthermore, to interpret certain facts of subsequent torsional development of the alimentary tract in the light of the growth of the epithelial tube in the manner of a left-handed helix and the reaction of the mesenchyme to this epithelial growth. The origin of the spiral epithelial growth is also briefly considered.

To an advocate of the experimental sciences it is undoubtedly necessary that an actual experiment should be made showing that by gradual stretching of a cell, under the requisite circumstances, it

is transformed into a muscle cell. To this end the writer is directing his attention. It must not be forgotten, however, that valuable suggestions pointing to a tensional stimulus as a factor in myogenesis is derived from a study of the origin of this tisue in a closely graded and advancing series of embryos. In the latter case direct observation reveals what is actually going on in nature's own laboratory.

Observations on the Early Development of the Esophagus of the Pig.

Hitherto, descriptions of esophageal development were written from a view-point regarding histogenesis as passive. No correlation of the developing epithelial tube and the surrounding mesenchyme has been presented. That one element could influence the other during the critical genetic steps has been overlooked. In order to comprehend clearly the development of the esophagus, or any formative structure for that matter, the active, dynamic point of view must be possessed by the observer and not the purely passive one. The interaction and interdependence of integers united in a common structure must be considered together and not as isolated, non-related entities. It is from the dynamic aspect then that the following observations differ from previous accounts of esophageal genesis. It is also to be noted that certain developmental gaps are herein filled, thereby making the sequence of histogenetic events objectively evident.

Corresponding portions of the middle of the esophagus from an ascending series of embryos were selected for comparative study. This region was primarily chosen due to the fact that the lower cervical and upper thoracic portions have become narrowed due to the elongation of the esophagus concomitant with the descent of the stomach. The diminution in the diameter of this zone, leads to a more rapid revolution of the epithelial mitotic figures on account of the decrease in circumference of the epithelial tube. This more rapid rate of rotation causes a corresponding greater vortical agitation in the less active fluid like mesenchyme. The reacting maelstrom is consequently more clearly seen and the path of the activating mitotic figures more easily followed. The muscular rim bordering the mesenchymal vortex is also more clearly defined.

The characteristic embryological facts will be presented in a closely graded series of pig embryos ranging from 9.5 to 24 mm. in length. It is during this short period that the genesis and growth of the inner, close spiral, smooth muscular coat is taking place. During the latter part of this period the outer elongated spiral or longitudinal muscular coat is becoming faintly indicated.

In a 9.5 mm. embryo (Fig. 1) the esophagus possesses a small epithelial tube composed of two to four rows of nuclei surrounding an oval or elliptical lumen. Enclosing the epithelial tube are found the undifferentiated mesenchymal cells. These are uniformly distributed in a spiral manner from the center to the periphery. The vortical arrangement of the mesenchyme together with growth of the esophagus in width and length depends upon the growth of the epithelial tube (Fig. 15).

The characters of the esophagus of pig embryos correspond closely to those found by F. T. Lewis and Johnson for human embryos of 8.4 to 16 mm. in length. Near the larynx it is crescentic in shape with the concavity of the crescent directed toward the trachea. This is a compression concavity due to tracheal growth. Caudad, the trachea first becomes round then transversely elliptical. In the upper thoracic region near the bifurcation of the trachea it is rounded but further caudad it merges with the stomach possessing a shape dorsoventrally elliptical.

The lumen is pervious and contains an albuminous liquid demonstrable in the living embryo. This liquid forms a reticular coagulum in fixed specimens (Figs. 1 to 6). The hydrodynamic influence of the liquid content found throughout the alimentary canal during mammalian development has been entirely neglected. In older embryos the accumulation of this intercellular fluid in isolated spaces which subsequently become confluent with the lumen is a mechanical aid in the separation and active moving apart of the cells resulting in enlargement of the lumen.

With further development the mesenchyme surrounding the esophageal tube becomes more discrete and defined from that enclosing the trachea. This is clearly seen in a 11.5 mm. embryo as shown in Fig. 2. The vortical arrangement of the cells is becoming more clearly marked. The mesenchymal cells show varying degrees of

FIGS. 1 to 6. Development of the esophagus and trachea at corresponding levels through the lower cervical region of pig embryos ranging in length from 9.5 to 24 mm. (1) trachea surrounded by a nebular mass of undifferentiated mesenchyme; (2) esophagus, surrounded by a nebular mass of undifferentiated mesenchyme; lumen contains reticular coagulum; (3) left vagus nerve; (4) right vagus nerve; (5) inner close spiral muscle; (6) outer elongated spiral muscle; (7) ganglionic cells of myenteric plexus (Auerbach's plexus); (8) ganglionic cells of submucous plexus (Meissner's plexus); (9) intercellular vacuoles of esophageal epithelium; (10) mesenchyme and embryonic cartilage of the ventral aspect of the trachea.

FIG. 1. Transverse section through the lower cervical region of a 9.5 mm. pig embryo.

FIG. 2. Transverse section through the lower cervical region of an 11.5 mm. pig embryo.

FIG. 3. Transverse section through the lower cervical region of a 14 mm. pig embryo.

FIG. 4. Transverse section through the lower cervical region of a 15 mm. pig embryo.

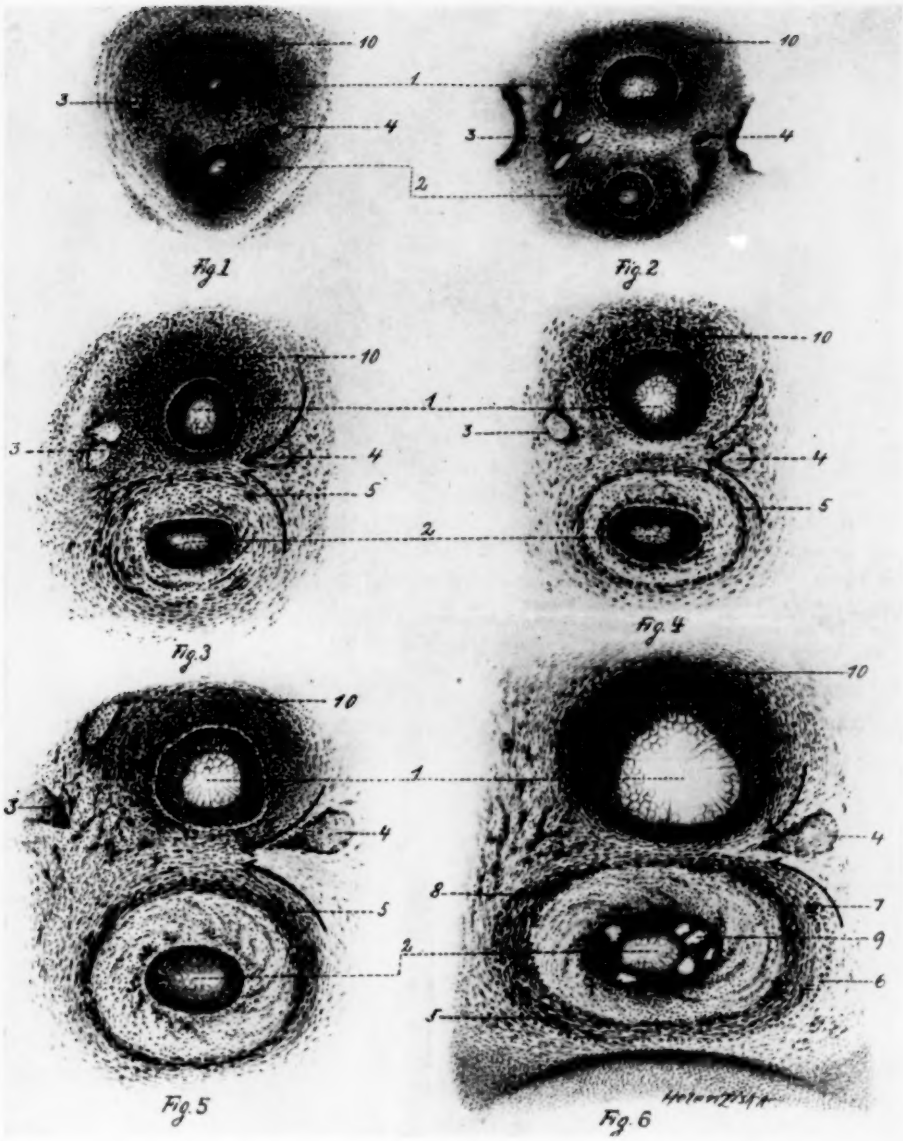
FIG. 5. Transverse section through the lower cervical region of an 18 mm. pig embryo.

FIG. 6. Transverse section through the lower cervical region of a 24 mm. pig embryo.

The more rapid growth of the tracheal epithelial tube over that of the esophagus is to be especially noted. The mesenchyme around the trachea also grows at a more rapid rate than that around the esophagus. Consequently the less rapidly growing esophageal mesenchyme is thrown into a veritable vortex. This vortex represents a centrifugal reaction of the mesenchyme to the centripetal action of the esophageal epithelial tube. This tube is rapidly growing in the manner of a left-handed helix. At the periphery of the mesenchymal whirlpool the optimum tensional stimulus is presented resulting in the histogenesis of the inner, close spiral, smooth muscle coat. The outer muscle coat is faintly detected in the esophagus of a 24 mm. pig embryo.

The mesenchyme around the trachea, due to its rapid growth is compressed. This compression results in the formation of cartilage tissue. The embryonic cartilage is becoming evident in the trachea of a 24 mm. pig embryo (Fig. 6).

The reacting mesenchyme tends to converge between the trachea and the esophagus on the right and diverge on the left. The right vagus nerve appears to be drawn between the two tubes by this action.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FIGS. 7 to 14. Cross-sections of the trachea and esophagus of a 9.5 mm. pig embryo. Note particularly that the mitotic figures of the trachea follow the path of a right-handed helix, whereas those of the esophagus follow the path of a left-handed helix. These two tubes, therefore, tend to rotate in opposite directions in development. This is comparable to the cylindrical rollers of a printing press. The mesenchymal vortices converge on the right side, represented by arrows, and diverge on the left. This is more evident in older specimens (see Fig. 6). (1) trachea; (2) esophagus; (3) proliferation bud of the trachea. These epithelial cells appear to be thrown off by an eruption and lost in the mesenchyme. At this location the basement membrane of the epithelial tube is absent. The rapid rate of growth of the tracheal epithelial tube probably causes this eruption by reacting centrifugal force.

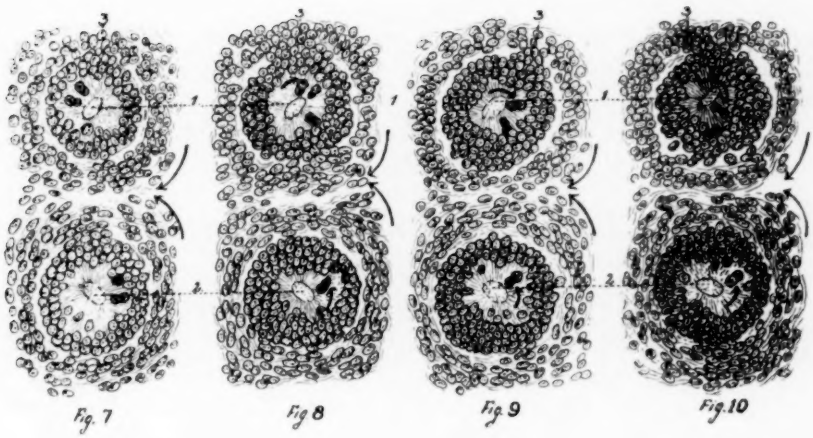

Fig. 7 Fig. 8 Fig. 9 Fig. 10

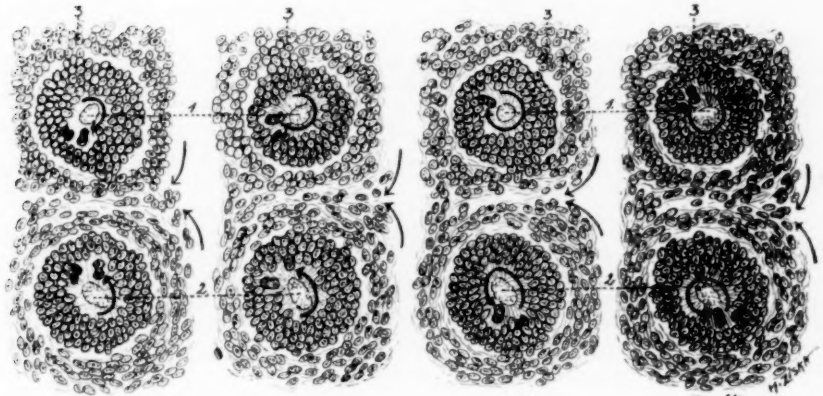

Fig. 11 Fig. 12 Fig. 13 Fig. 14

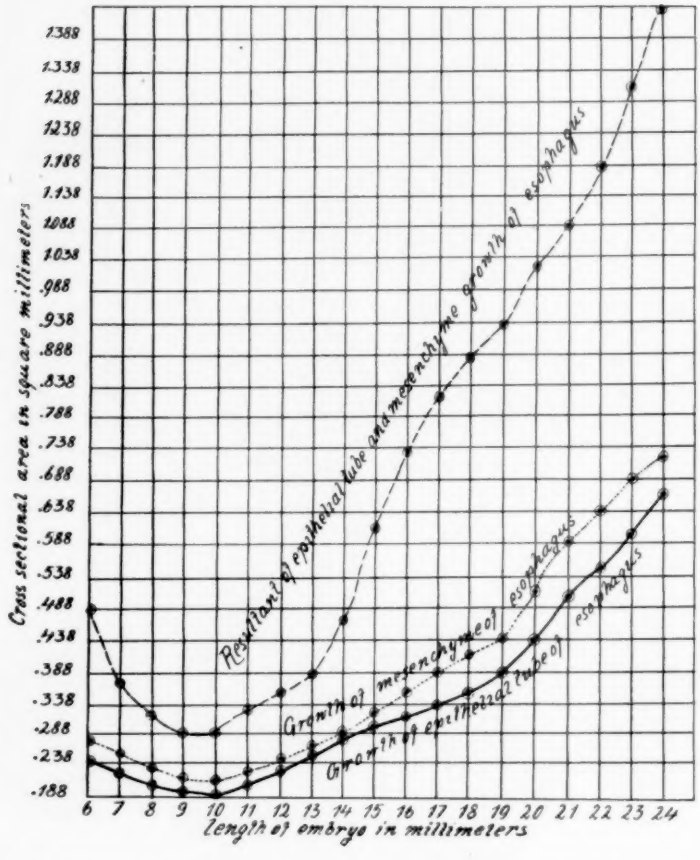

FIG. 15. Curves of differential growth of the epithelial tube and mesenchyme of the esophagus. Since there is no restricting peritoneal membrane in the lower cervical region of the esophagus the more rapid rate of growth of the epithelial tube over that of the mesenchyme is not as definite as that seen in the descending colon. The great extent of mesenchymal area is due to the centripetal force of the epithelial tube. The centrifugal reaction tends to throw the cells off tangentially. Consequently, the greater area of the mesenchyme is not due to an intrinsically more rapid rate of mitosis over that found in the epithelial tube but to the radial force (centrifugal force) of the mesenchyme in reaction to the centripetal force of the epithelial tube.

elongation. Those at the periphery are more stretched than those centrally located. The nuclei are first elongated in the direction of the epithelial growth and shortly thereafter granules are found in the intercellular and intracellular cytoplasm. These granules are arranged in rows, likewise in the direction of the growth of the epithelial tube. Yet the distribution of the cells is fairly uniform throughout. No cell boundaries are presented. The entire region is a syncytium studded with nuclei surrounded by a narrow zone of granular endoplasm. The ectoplasm forms the connecting phase which characterizes the unity of organization found in the mesenchyme.

The epithelial tube, larger in the 11.5 mm. pig embryo than in the 9.5 mm. pig embryo (Fig. 1), possesses an increased number of cells. Numerous mitotic figures may be plotted in serial sections (Fig. 5). These form the path of a definite left-handed helix from above downward (Figs. 7 to 14).

The left-handed helix of the large intestine is also followed by the epithelial mitosis but from below upwards. The ileocecal valve is one point of convergence of the helicoidal growth of the epithelial tube. The increased growth in diameter of the large intestine with the path of mitosis directed cephalad meets the smaller diameter of the ileum with its mitotic path directed caudad. These are mechanical factors involved in the formation of the definite valve-like constrictors in the alimentary tract.

As development continues to the stage of a 14 mm. embryo there is a rapid increase in size of the epithelial tube. The lumen, although larger, is not correspondingly as large as the entire tube. The increase in size of the latter, therefore, is due to an increase in the number of cells crowding the lumen.

Concomitant with the rapid growth of the epithelial tube a beginning of formation of the apparent ring of stretched cells is found at the periphery of the mesenchymal vortex arranged in a tangential position. Various degrees in the elongation of the mesenchymal cells are found decreasing in intensity of stretching from without inwards.

On the outer side of the myoblastic ring the intercellular substance is drawn out into myofibrillæ. On the inner side of the ring, discrete granules are arranged in the same tangential direction as the elongated nuclei of the myoblasts. That the same force produces these

results in the differentiation of muscle tissue is evident. These differential elements are elongated or arranged in the same direction around the ring. These structures represent the tensional reaction of the mesenchyme to the force of epithelial tubular growth.

The apparent annular formation of the mesenchymal cells at the periphery of the vortex is fairly established in an embryo 15 mm. in length (Fig. 4). At a distance the ring appears discrete but when examined with higher magnifications, there is revealed a gradual transition and blending of the degrees of stretching as the cells are examined from the periphery toward the central epithelial tube. The cells which have been subjected to the optimum degree of stretching for muscle origin at the periphery of the mesenchymal whirlpool react by forming myoblasts. Those more centrally placed have been evidently subjected to the optimum tension for the formation of embryonal, connective tissue. From direct observation it appears that the formation of a specific derivative from a pluripotent, mesenchymal cell is due to the fortuitous circumstance of position. Those cells at the periphery of the mesenchymal maelstrom form muscle, those toward the center form embryonal connective tissue.

The esophagus of an 18 mm. embryo (Fig. 5) shows still more clearly the inner, close spiral musculature, forming an apparent ring. Internal to the ring are found ganglionic clusters of the submucosa or Meissner's plexus; externally are found similar clusters and ramifications of the vagus nerve forming the myenteric or Auerbach's plexus. These are first detected in embryos 12 to 14 mm. in length.

The submucous, embryonal connective tissue interposed between the musculosa and epithelial tube contains numerous capillaries which by injection methods are demonstrated to pursue a spiral course. These capillaries form from discrete vesicles by confluences at the 15 mm. stage. With the development of the inner, close spiral musculature the connective tissue is still more definitely found to be arranged in spirals.

In the 24 mm. pig embryo (Fig. 6) the external elongated spiral or longitudinal muscle coat is becoming faintly indicated. This is more clearly seen in longitudinal than in cross-sections. This formation is taking place concomitant with the relative and absolute increase of the ratio of the length over that of the diameter of the esophagus.

The precocious segregation of the esophageal epithelial cells would appear to be distinctly disadvantageous, for here a much larger proportion of each cell is in contact with others than in the looser texture of the mesenchyme. The intercellular vacuoles filled with fluid seen in the epithelium of the esophagus of a 24 mm. pig embryo (Fig. 6) is a provision for aiding excretion. These intercellular vacuoles are comparable to the periodic appearance of intercellular cavities observed by Kofoid in *Limax*.

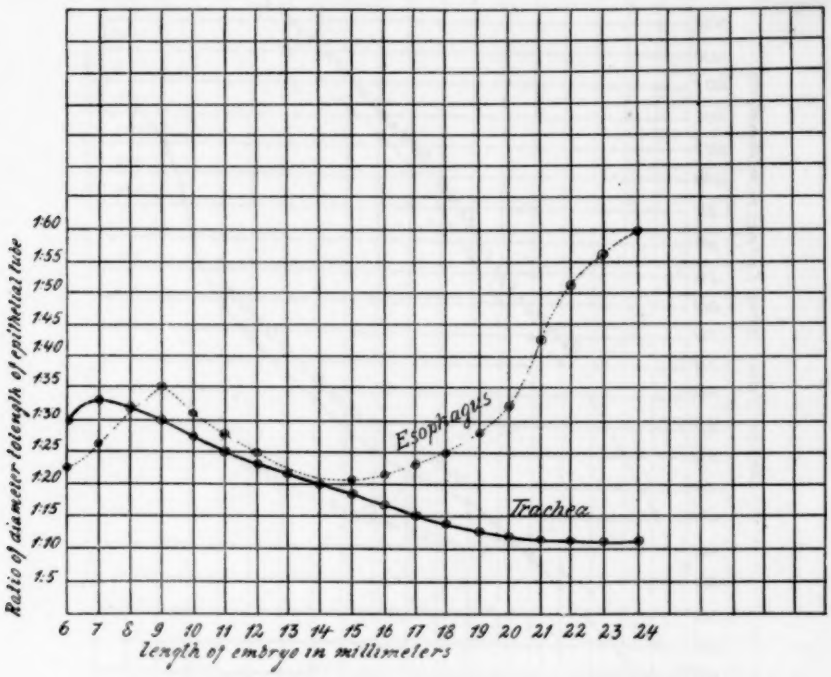

FIG. 16. Curves of ratio of diameter to length of the epithelial tubes of the esophagus and trachea. Note particularly that the esophageal tube grows relatively more rapidly in width in embryos 10 to 15 mm. in length, whereas, in embryos from 15 to 24 mm. in length the epithelial tube grows relatively more rapidly in length. In the former period the inner, close spiral, muscle coat is differentiating; toward the end of the latter the outer, elongated, spiral coat is being formed. The relatively greater growth in width than in length of the tracheal epithelial tube is objectively evident. This rapid growth is the efficient cause in compressing the tracheal mesenchyme, acting as the stimulus in the differentiation of the tracheal cartilage.

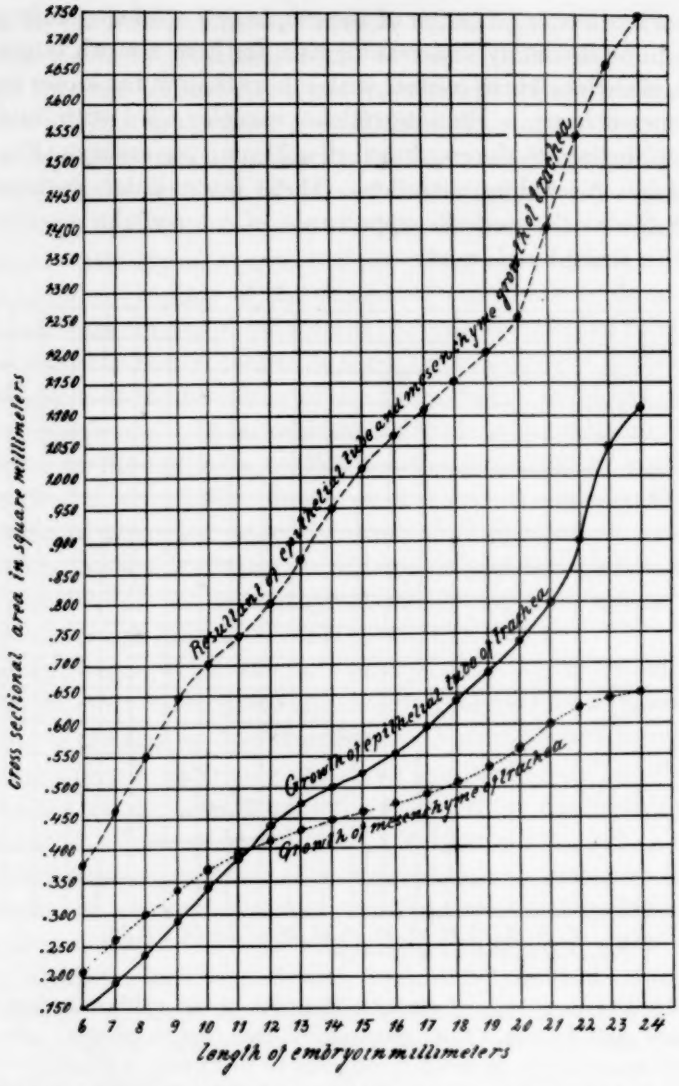
cross sectional area in square millimeters
length of embryo in millimeters
Resultant of epithelial tube and mesenchyme growth of trachea
Growth of epithelial tube of trachea
Growth of mesenchyme of trachea

FIG. 17. Curves of differential growth of the tracheal epithelial tube and mesenchyme. The greater growth of the epithelial tube over that of the mesenchyme after the embryo has reached a length of 11 mm. is shown graphically. This rapid growth causes a progressive compression of the mesenchyme acting, therefore, as the efficient stimulus in the differentiation of cartilage tissue. The tracheal embryonic cartilage appears in embryos between 18 and 24 mm. in length. Note particularly that during this period the curve of epithelial tubular growth in area rapidly ascends.

In the earlier stages the esophageal epithelial tube grows relatively more rapidly in diameter than in length (Figs. 16 and 17). In the 9.5 mm. embryo the esophageal epithelial tube measures 0.04 mm. in diameter and 1.3 mm. in length; in the 14 mm. embryo the diameter is 1 mm. and the length 2.1 mm. Therefore, the esophageal epithelial tube is growing relatively more rapidly in width than in length, from the 9.5 to 14 mm. embryonic stages.

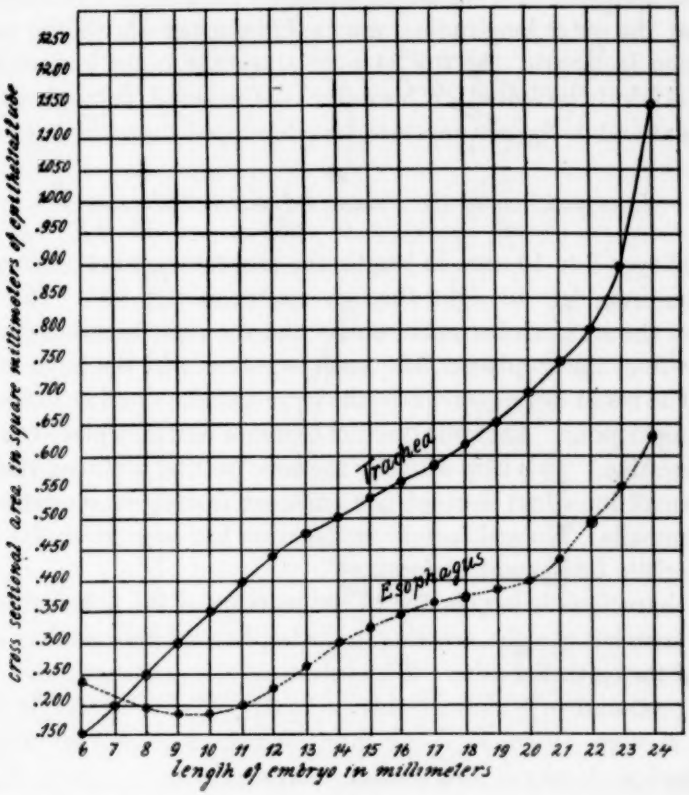

FIG. 18. Curves of differential growth in area of the tracheal and esophageal epithelial tubes. These curves show strikingly the more rapid growth in the same unit of time of the tracheal tube over that of the esophageal tube. Note that between embryos 6 to 10 mm. in length there is an absolute decrease in area of the tube. This decrease is concomitant with the rapid descent of the stomach. This decrease in area is due to stretching as the result of traction caused by the descent of the stomach. After the stage of the 10 mm. embryo, growth in area of the esophagus rapidly progresses.

The reverse relation takes place, however, in embryos ranging in length from 14 to 24 mm. (Figs. 16 and 18). In the 24 mm. embryos the esophagus measures 9.1 mm. in length and 0.15 mm. in diameter. We see, therefore, that the length increases four times over that found in the 14 mm. embryo, whereas the diameter only increases one-half. These facts help us to coordinate intelligently, for it is now evident that the inner close spiral musculature is in the process of formation while the epithelial tube is growing relatively more rapidly in diameter and that the outer longitudinal muscle is incepted during the rapid elongation in length. At the 24 mm. stage the outer longitudinal muscle is faintly indicated. This outer coat is found, throughout development and in the mature state, less developed than the inner one.

Interpretation of the Primary Intestinal Torsion.

In embryos 5 to 10 mm. in length the alimentary tract undergoes a torsion from left to right through approximately 90°. The left aspect of the stomach becomes ventral and the right becomes dorsal. This involves the esophagus, the small intestine, and the large intestine. The result of this torsion in the large and the small intestines is a reacting looping. The colic fraction becomes ventrally placed to the small intestine. This loop whereby the colic portion is placed ventral to the small intestinal element is a constant fundamental relation in the Mammalia. No satisfactory explanation has been given for this characteristic fundament of form.

This characteristic loop is due to the reaction of the elastic mesenchyme, from left to right, reacting to the left-handed helicoidal growth action of the epithelial tube. With intestinal elongation an inevitable loop is produced in a characteristic manner. This is clearly demonstrable in Figs. 19 to 24. The intestino-colic flexure, therefore, is a mechanical torsional reaction of the mesenchyme. This right-handed helicoidal reaction is due to the left-handed helicoidal growth of the epithelial tube.

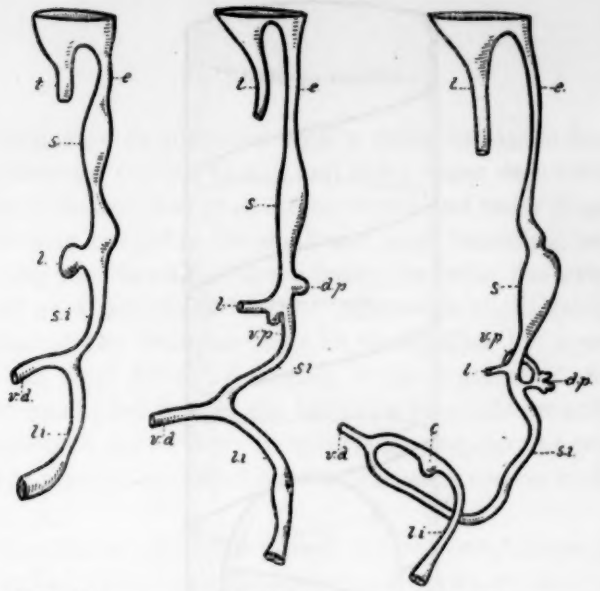

FIG. 19. FIG. 20. FIG. 21.

FIG. 22. FIG. 23. FIG. 24.

FIG. 19. Schema of digestive tract of a 6 mm. pig embryo.

FIG. 20. Schema of digestive tract of an 8 mm. pig embryo.

FIG. 21. Schema of digestive tract of a 10 mm. pig embryo.

FIG. 22. Schema of elastic band twisted as a left-handed spiral. This represents the mesenchyme of the straight digestive tract.

FIG. 23. Schema of ventral flexure of an elastic band; represents the ventral flexure of elongating tract of Fig. 20.

FIG. 24 Schema of inevitable ventral looping of caudal element over the cephalic element of an elastic band twisted as a left-handed spiral. This represents the reaction of the mesenchyme to the torsion produced by the spirally growing epithelial tube as schematized in Fig. 21. The loop tends to relieve the torsion to which the mesenchyme is subjected.

t, trachea; *e*, esophagus; *s*, stomach; *l*, liver; *d. p.*, dorsal pancreas; *v.p.*, ventral pancreas; *s.i.*, small intestine; *c.*, cecum; *v.d.*, vitelline duct.

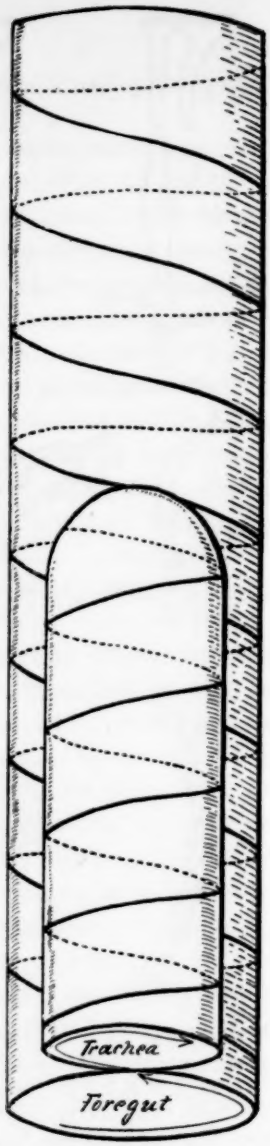

FIG. 25. Diagram of the tracheal bud from the ventral aspect of the foregut. The former presents mitotic figures following the path of a right-handed helix. The latter presents mitotic figures following the path of a left-handed helix. The tracheal or lung bud is an outgrowth from the ventral aspect of the cephalic aspect of the entodermal foregut. The cause of the mitosis pursuing a path of a right-handed helix in the trachea is due to the deflection of the left-handed, helicoidal, mitotic path of the foregut. This deflection occurs at the cephalic orifice of the tracheal epithelial tube where it arises from the floor of the entodermal tube of the foregut.

This loop may be produced with a string or elastic band. With one end between the left thumb and index finger directed above and the lower element between the right thumb and index finger directed below we may exemplify the small and large intestines, respectively. By rotating the thumb and index finger clockwise, the string or band is wound as a left-handed helix. After this slight initial torsion, elongation of the intestine may be exemplified by approximating the left and right hand. A looping of the segment of the string in the right hand over that of the left is an inevitable reaction due to the direction of the torsion. A reverse looping may be produced by winding in the opposite direction or by excessive torsion in the previous direction.

The direction of epithelial growth is counterclockwise or as a left-handed helix. The elastic mesenchyme is drawn out in a corresponding direction. The resultant reaction of the mesenchyme is in the reverse direction from left to right. This is an inevitable reaction of the mesenchyme to the active torsion produced by the dominant epithelial growth. The torsion of the digestive tube, therefore, is a mesenchymal reaction to the torsional action of growth of the epithelial tube.

Situs Inversus Viscerum.

The normal asymmetry of the abdominal viscera as well as the position of the gut is dependent upon the action of the spiral growth of the epithelial tube and the resultant reaction of the mesenchyme. The left-handed helicoidal growth of the epithelium causes a corresponding arrangement of the cells of the less actively growing mesenchyme. The cells of the latter are elongated or stretched. Subsequently these mesenchymal cells react in the opposite direction from left to right. This causes the 90° rotation of the gut in a clockwise direction. The ventral position of the liver bud prior to rotation is now directed to the right comparable to the 90° displacement dorsally of the right vagus nerve after rotation is completed.

Pressler produced experimentaly *situs inversus* in *Bombinator*. While the embryo was in the neurula stage Pressler exsected a quadrilateral piece of the medullary plate plus a part of the dorsal surface of the primitive gut. This piece was then replaced in the reversed

position so that the cephalic end was directed caudad and the caudal end was directed cephalad. Tadpoles were reared from these experimental embryos and in many cases a complete *situs inversus viscerum* was presented.

The transposition of the viscera is due to the fact that the reversed segment presents a screw-like growth action of the epithelium opposite to that normally present. The mesenchymal cells form a right-handed spiral arrangement and in reacting to stretching cause a 90° rotation of the gut from right to left, or counterclockwise. This reversed rotation due to the torsional reaction of the mesenchyme would cause the ventrally placed liver bud to be directed to the left instead of to the right.

An excellent discussion of the views of Wilder, Bateson, and Newman as regards mirror imaging and symmetry reversal in monsters will be found in a paper by Morrill. That Morrill had a clear-cut perception of the location in which solution of the problem of normal asymmetry of the viscera was to be found is seen in the following statement: "the factors controlling asymmetry are located in the primitive gut and become operative before the liver bud has developed."

Crampton suggested that asymmetry in the viscera may depend upon spiral cleavage during the first stages of development. This rested on the fact that the majority of snails possess a dextral shell associated with a right-handed spiral cleavage. Certain snails have a sinistral shell and reversed asymmetry in the viscera. This reversal of the viscera was associated with a left-handed spiral cleavage.

From a study of vertebrate monsters, however, Morrill correctly comes to the following conclusion: "From the evidence at hand, it seems probable that the primary cause of visceral asymmetry in vertebrates is to be sought for at the completion of cleavage rather than in the period of cleavage itself."

Origin of Spiral Growth.

The explanation of spiral epithelial growth is under investigation. Spiral growth involves two motions: a motion of rotation, circular, and a motion of straight progression, rectilinear. The latter is evidently imparted from growing points of mitosis. Growth would

tend to pursue a straight line if the factors of resistance due to differential growth did not interfere. An exception to this would be found in the spiral type of cleavage observed by Child in *Arenicola cristata*, and Wilson in *Nereis*. In these forms the rotation of the cells appears to be predetermined in the parent cells, as is proved by the position of the spindles and by the form of division. In the intestine the inner curved surface of the mesenchymal wall constantly deflects the mitotic path in a circular manner. This would impart the rotary element to the compound spiral motion of growth.

The relation of the pharynx to the esophagus and of the rectum to the colon is comparable to that of the wide and narrow components of a funnel. A stream running down the inclined wall of the wide element would be deflected in a rotary manner so that its progression through the narrow integer would be in the manner of a helix. The determination of a left- or right-handed helix would depend upon the initial direction of impelling the stream.

The direction of epithelial growth in a left-handed helix has a definite mechanical basis in the intestine, but as yet is undetermined. The epithelial growth is roughly comparable to the stream exemplified above; *i.e.*, the glass of the funnel to the mesenchyme. The initial impulse of the water pressure is comparable to that of growth. This causes the rectilinear motion of progression. The circular resistance of the glass is analogous to that of the mesenchyme. This causes a deflection of the rectilinear motion in a rotary manner, the two characterizing the helicoidal motion of the intestine.

CONCLUSIONS.

Esophageal Development.

1. The region of most active mitosis per mm. of cross-section in the esophagus is the entodermal epithelial tube. The mitotic figures follow a spiral path in the manner of a left-handed helix from the cephalic to the caudal direction.

2. The region of least active growth per mm. of cross-section in the esophagus is the mesenchyme surrounding the epithelial tube.

3. The helicoidal activity of the epithelial tube causes a vortical reaction in the surrounding mesenchyme. The mesenchymal whirlpool represents a reaction to the spirally growing epithelial tube.

4. In embryos 9.5 to 14 mm. in length the esophageal epithelial tube grows relatively more rapidly in width than in length. During this period the myoblasts which form the inner, close spiral, muscle coat of the esophagus are becoming rapidly differentiated in the outer condensed margin of the mesenchymal maelstrom.

5. The nuclei, first spherical then oval, and finally rod shaped with rounded ends, are drawn out in the direction of the circumference of the mesenchymal rim which is directed tangentially.

6. The cytoplasm is also drawn out in the direction of the mesenchymal rim of the vortex. The elongated rows of isolated granules appear which subsequently, by confluence, form the myofibrillæ. These cytoplasmic derivatives are elongated in the direction of the circumference of the vortex.

7. Between the epithelial tube and the myoblastic rim at the periphery of the mesenchymal whorl is found the embryonic connective tissue. From this direct observation the conclusion is made that an optimum tensional stress stimulus is necessary to elicit the formation of muscular tissue at the circumference of the mesenchymal vortex. Consequently, the formation of a specific derivative from a pluripotent mesenchymal cell is due to the fortuitous circumstance of position.

8. In embryos from 14 to 24 mm. in length, the esophagus grows relatively more rapidly in length than in width. This elongation is due to two factors; first, the descent of the stomach, and, second, the resistance to diametrical growth presented by the inner close spiral musculature. The epithelial tube, still the dominant zone of mitotic activity, pursues the lines of least resistance, and consequently growth in length takes place. This is due to the shifting of the planes of cell division on account of the compression of the inner, close spiral, muscle coat.

9. The undifferentiated mesenchyme peripherad to the inner, close spiral musculature is elongated and the histogenetic changes in muscular formation are gradually taking place between 14 and 24 mm. A very attenuated, outer, elongated, spiral, or longitudinal muscle coat is detected in the esophagus of a 24 mm. pig embryo.

10. The characteristic intestino-colic flexure is a torsional reaction of the mesenchyme. The mesenchymal cells are thrown into a left-handed helicoidal series, corresponding to the activity in the epithelial

tube. The right-handed helicoidal reaction of the mesenchyme, therefore, is due to the left-handed helicoidal growth of the epithelial tube.

11. The normal asymmetry of the abdominal viscera as well as the position of the gut is dependent upon the clockwise reaction of the stretched mesenchymal cell. These cells are stretched by the left-handed helicoidal growth of the epithelial tube. One factor producing *situs inversus viscerum* could be the reversal of the spiral growth of the epithelial tube resulting in a reaction of the mesenchyme in a direction opposite, namely counterclockwise, to that which occurs normally.

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ION SERIES AND THE PHYSICAL PROPERTIES OF PROTEINS. I.

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I. INTRODUCTION.

It had been shown in a preceding paper[1] that the relative influence of ions on the rate of diffusion of water through collodion membranes can be expressed by arranging the ions in two definite series, as follows:

(1) Rb < K < Na < Li < divalent < trivalent cations.

(2) Cl < Br < I < divalent < trivalent anions.

These results are intelligible on the assumption that we are dealing in this case with electrostatic effects of ions, the electrostatic effects of the cations being due to the excess charges of the positive nucleus and the electrostatic effects of the anions being due to the charges of the captured valency electrons. In this case the relative efficiency of monatomic and monovalent cations should increase inversely with the radius of the ion; and the relative efficiency of the monovalent, monatomic anions should increase directly with the radius of the ion. In the cation series the Li ion should be next to the divalent cations, while in the anion series the iodion should be next to the divalent anions.

This order differs from that usually given for the action of ions on the physical properties of proteins. Hofmeister[2] and afterwards Pauli[3] determined the relative order of efficiency of ions for the precipitation of proteins. This order is according to Pauli:

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 673.

[2] Hofmeister, F., *Arch. exp. Path. u. Pharm.*, 1888, xxiv, 247.

[3] Pauli, W., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 225; *Fortschr. naturwiss. Forschung*, 1912, iv, 237.

For cations, $Mg < NH_4 < K < Na < Li$.

For anions, CNS < I < Br < Cl < acetate < tartrate < citrate < phosphate < sulfate < Fl.

The reader will notice that in the cation series the divalent cation Mg is next to NH_4 and K, while we should expect it to follow Li in order of efficiency. On the other hand, if the efficiency of the monovalent, monatomic cations increases in the order of K < Na < Li as Hofmeister and Pauli observed we should expect the efficiency of the anions to increase in the order of Cl < Br < I instead of in the reverse order. We must conclude that the phenomena of precipitation of proteins by ions are either not determined by the electrostatic forces of the ion or that other variables enter which are not yet known. This latter possibility exists since the concentration of electrolytes required for precipitation is very high and the writer has shown that the influence of ions causing the anomalous diffusion of water through membranes already ceases to be noticeable in comparatively low concentrations of electrolytes.[4]

It therefore seems advisable to restrict our attention to such influences of ions where they act in low concentrations. This is possible when we compare the effects of low concentrations of different acids and alkalies on the physical properties of proteins. The first one who undertook such a study on a larger scale was Pauli. He recognized that in order to investigate the effect of acids and alkalies on proteins it was necessary to free them first from salts. When Pauli and Handovski[5] added to blood albumin dialyzed for 6 weeks various quantities of different acids they found that all the acids increased the viscosity of the blood albumin but in a different degree. Their results are expressed in curves from which we gather that the relative order of efficiency of various acids tried by them was

HCl > monochloracetic > oxalic > dichloracetic > citric > acetic > sulfuric > trichloracetic acid

where HCl raises the viscosity most, and trichloracetic least. The viscosity was measured by the time of outflow through a viscometer. Pauli assumes, as Laqueur and Sackur[6] had done previously in their

[4] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.

[5] Pauli, W., and Handovski, H., *Biochem. Z.*, 1909, xviii, 340.

[6] Laqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.

experiments on casein solutions, that the increase in the viscosity of the protein caused by the addition of acid (or of alkali) is due to an increase in the ionization of proteins, as a consequence of the salt formation between protein and acid or alkali, the protein salt being capable of a greater degree of ionization than the protein not treated with acid or alkali. In order to explain the relative differences in the effect of various acids on the viscosity, Pauli assumes that the protein salts formed with different acids differ in their degree of electrolytic dissociation. He states; *e.g.*, that "the strong trichloracetic acid and the slightly weaker sulfuric acid furnish few protein ions."[7] He also states that the relative efficiency of different anions on the other properties of proteins, as osmotic pressure, precipitation by alcohol, is the same as that found for viscosity.

In his experiments on the influence of acids and alkalies on the osmotic pressure of gelatin solutions the writer had arrived at the conclusion that the results he obtained cannot be expressed in terms of ion series.[8] These experiments showed only an influence of the valency of the anion or cation in combination with the gelatin, but no other influence. Solutions of Li, Na, K, and NH_4 gelatinate of the same pH and the same concentration of originally isoelectric gelatin had the same osmotic pressure. The same was true for Ca and Ba gelatinate; but the osmotic pressure of Ba and Ca gelatinate was only one-half or less than that of Li, Na, K, or NH_4 gelatinate of the same pH and the same concentration of originally isoelectric gelatin. The influence of acids on the physical properties of proteins was still more interesting. Solutions of gelatin chloride, bromide, nitrate, acetate, phosphate, citrate, tartrate, succinate, of the same pH and the same concentration of originally isoelectric gelatin, had approximately the same osmotic pressure, while the osmotic pressure of solutions of gelatin sulfate was only half or less than half of that of gelatin chloride, etc. The osmotic pressure of solutions of gelatin oxalate was almost but as a rule not quite as high as that of gelatin chloride.[8]

This peculiarity found its explanation in an investigation of the combining ratios of these acids with gelatin. The writer was able to

[7] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 243.
[8] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483, 559.

show that the strong dibasic acid H_2SO_4 combines in equivalent proportions with gelatin while the weaker acids, *e.g.* phosphoric or oxalic, combine in molecular proportions. At a given pH, *e.g.* pH = 3.5, 1 gm. of originally isoelectric gelatin is in combination with three times as much 0.1 N acid when the acid is H_3PO_4 than when it is HCl; and with almost twice as much 0.1 N acid when the acid is oxalic than when it is HCl; while the ratio of HCl and H_2SO_4 is 1. It follows from this that the strong dibasic acid H_2SO_4 forms a salt with gelatin in which the anion is divalent, namely SO_4, while the weak tribasic acid H_3PO_4 forms a salt with gelatin in which the anion is monovalent; namely, H_2PO_4 (instead of the trivalent anion PO_4). Likewise, citric, succinic, and tartaric acids form gelatin salts in which the anion is always monovalent; namely, H-succinate, H-tartrate, etc. In the case of oxalic acid this is also the case though a slight amount of gelatin salt with divalent anion is probably formed and this might account for the fact that the osmotic pressure of gelatin oxalate is generally slightly less than that of gelatin chloride.[9]

It was also found that $Ca(OH)_2$ and $Ba(OH)_2$ combine with gelatin in equivalent and not in molecular proportion thus showing that the cation of Ca gelatinate and Ba gelatinate is divalent.[8]

All these facts together show that solutions of salts of gelatin with a bivalent ion have an osmotic pressure of one-half or less of that of solutions of salts of gelatin with monovalent ions at the same pH and the same concentration of originally isoelectric gelatin.

This then proves that while the valency of the ion in combination with the gelatin has a strong influence on the osmotic pressure of the gelatin solution differences in the nature of ions of the same valency have either no effect on the osmotic pressure or if they have such an effect it is too slight to be noticeable by our method of experimentation. We can certainly say that it is impossible to express our observations in terms of the Hofmeister series. In the Hofmeister series phosphates and chlorides stand at almost opposite ends of the anion series while in our experiments the effects of H_3PO_4 and HCl are identical if we compare the effects at the same pH of the gelatin solutions. The reason that Pauli arrived at a different conclusion lies

[9] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483.

probably in the fact that he compared the effects of the addition of equal quantities of acid to a protein while we compared the effects of different acids at equal hydrogen ion concentrations of the solutions.

II. Action of Weak and Strong Monobasic Acids.

We will first indicate why it is necessary to choose equal hydrogen ion concentrations as a standard of comparison instead of using equal quantities of acid. We always use as standard material isoelectric protein, to which we add enough acid or alkali to bring it to the desired hydrogen ion concentration. In the case of gelatin we proceed usually in this way; we add to 1 gm. of powdered gelatin brought to the isoelectric point (pH = 4.7) small quantities of acid to bring the samples to the desired hydrogen ion concentration, then melt the gelatin, and bring the volume of the solution to 100 cc. by adding H_2O. The pH is then determined. It is hardly necessary to state that it requires greater quantities of weak than of strong acid to bring the gelatin to the same pH on the acid side of the isoelectric point. Fig. 1 gives the quantities of 0.1 N acetic, monochloracetic, dichloracetic, and trichloracetic acids required to bring 1 gm. of isoelectric gelatin in a 1 per cent solution to the same pH. The abscissæ in Fig. 1 are the pH of the gelatin solution resulting from the addition of acid and the ordinates are the numbers of cc. of 0.1 N acid which must be contained in 100 cc. of a 1 per cent solution of originally isoelectric gelatin to produce the pH. The curve indicates that the quantity of acid required is the less the stronger the acid. If we now measure the osmotic pressure of the solutions of the four gelatin acetates (all 1 per cent in regard to the originally isoelectric gelatin) and if we plot the curves with the values for the osmotic pressure as ordinates and the pH as abscissæ, the curves for all four gelatin-acetate salts are practically identical (Fig. 2). All four curves have a minimum at the isoelectric point pH = 4.7; they all rise identically with a diminution in pH (*i.e.* a rise in hydrogen ion concentration); all reach their maximum at a pH of about 3.5, and all drop almost as steeply with a further diminution of pH as they rise on the ascending side of the curve. The drop will not be discussed in this paper.

The slight differences in the maximal height of the four curves are within the limits of the accuracy of these experiments, and the same or even greater variations may be observed when the same experiment is repeated several times with the same acid. The maximal values of osmotic pressure reached in these experiments at pH 3.5 are the same as those reached with gelatin salts of any other monovalent anion, Cl, Br, NO_3, phosphate, citrate, succinate, etc.

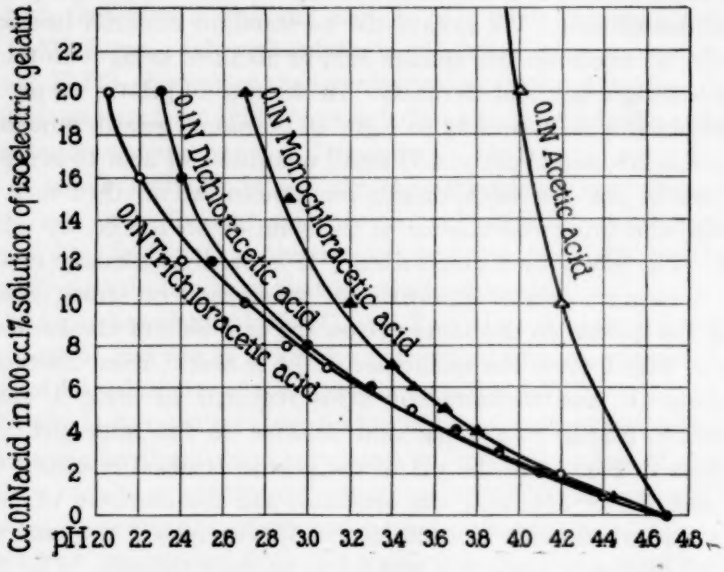

FIG. 1. The ordinates represent the number of cc. of 0.1 N acetic, mono-, di-, and trichloracetic acids required to bring 1 gm. of isoelectric gelatin to the pH indicated by the abscissæ. Enough H_2O was added to bring the gelatin-acid solution to a volume of 100 cc.

It follows from this that the conclusions which are based upon a comparison of the quantities of the acid added instead of upon the pH of the protein solutions cannot be correct.

The reason why we get identical curves when we plot the osmotic pressures as ordinates over the pH as abscissæ seems to be as follows.

When we add a definite small quantity of acid to 1 gm. of isoelectric gelatin, melt, and bring the volume of the solution to 100 cc. by adding H_2O, part of the acid will combine with gelatin and part will remain

free in the solution. The more acid we add, the greater the amount of isoelectric gelatin transformed into gelatin-acid salt. We assume that there is a definite equilibrium between the hydrogen ion concentration of the solution, the amount of gelatin-acid salt formed, and isoelectric gelatin. If this view is correct, it follows that at the same

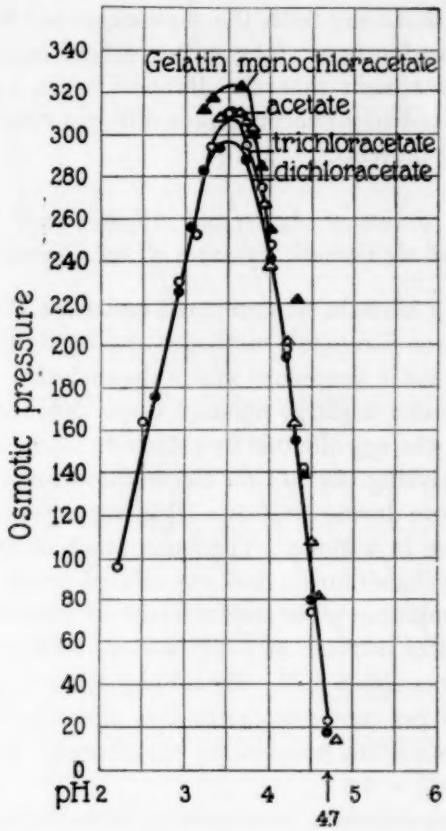

FIG. 2. The ordinates indicate the osmotic pressure (in mm. of the height of a column of the gelatin solution) of 1 per cent solutions of originally isoelectric gelatin which have been brought to different pH by the addition of the acids used in Fig. 1. The abscissæ are the pH. The curves are practically identical, the slight differences at the summit being inside the range of the variation found for the same acids.

pH and with the same concentration of originally isoelectric gelatin all mixtures of acid and gelatin solution must have the same concentration of gelatin-acid salt; and if this is the case they must all have the same osmotic pressure if the valency of the anion is the same, since the osmotic pressure is almost entirely determined by the gelatin-acid salt, that of the isoelectric gelatin being very low. This conclusion is in harmony with the view expressed by Laqueur and Sackur, Pauli, and others. The writer differs from Pauli only in regard to the statement that the different acids, *e.g.* HCl, H_3PO_4, tartaric, acetic, and trichloracetic, have different effects on the physical properties of proteins.

III. Combining Ratios of Acids and Alkalies with Crystalline Egg Albumin and the Osmotic Pressure of the Albumin Solutions.

Crystalline egg albumin was prepared under Dr. Northrop's supervision according to Sörensen's method,[10] and crystallized three times. The only difference in procedure was in the dialysis. Instead of putting the water under negative pressure as was done by Sörensen, pressure was put on the egg albumin by attaching a long glass tube full of water to the dialyzing bag so that the solution was under about 150 cm. water pressure during dialysis. This was necessary to avoid too great an increase in volume. The same stock solution of albumin served for all the experiments and was diluted before the experiment to a 1 per cent solution. The concentration of ammonium sulfate left in the solution was between M/1,000 and M/2,000. The pH of the stock solution was about 5.20. By adding about 1 cc. of 0.1 N HCl to 100 cc. of a 1 per cent solution of this albumin the solution was brought to the isoelectric point of the egg albumin, which is according to Sörensen at pH = 4.8.

The 1 per cent solutions were made up with different quantities of acid (or alkali) and the pH of the albumin solution was determined electrometrically.[11] In Fig. 3 are plotted the curves in which the pH are the abscissæ and the cc. of 0.1 N acid required to obtain the various

[10] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915–17, xii, 1.

[11] The colorimetric determination which gives fairly good results in the case of gelatin is unreliable in the case of egg albumin.

pH as ordinates. The curves represent these values for four acids, HCl, H_2SO_4, H_3PO_4, and oxalic acid. Beginning with the lowest curve we notice that the curve is the same for 0.1 N HCl and 0.1 N H_2SO_4, since both are strong acids; or, in other words, H_2SO_4 combines

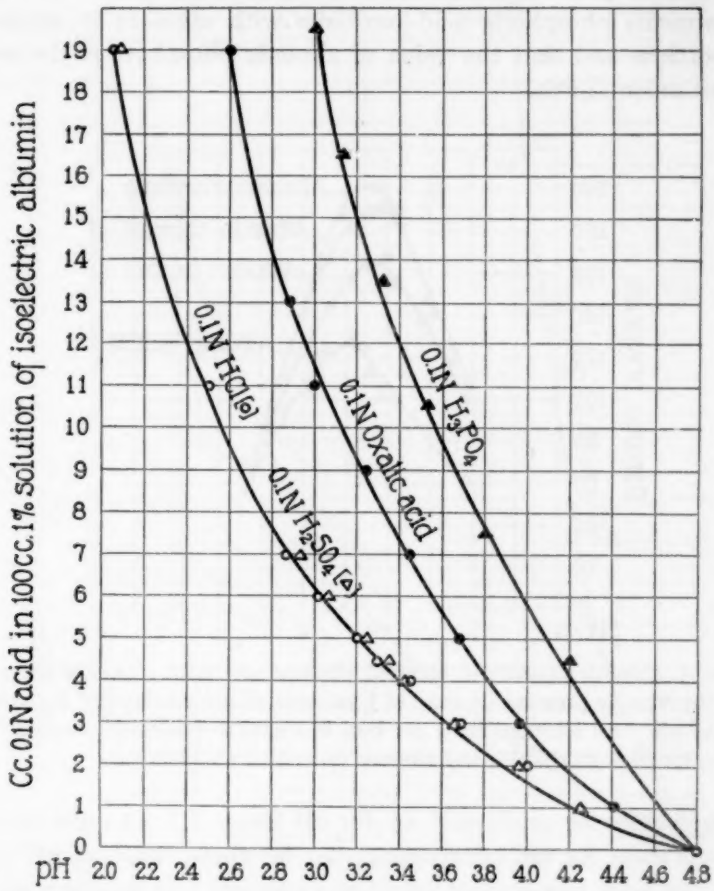

FIG. 3. The ordinates represent the number of cc. of 0.1 N HCl, H_2SO_4, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric crystalline egg albumin to the pH indicated on the axis of abscissæ. Enough H_2O was added to bring the solutions of albumin and acid to a volume of 100 cc. For the same pH the ordinates for HCl, H_2SO_4, and phosphoric acid are approximately as 1 : 1 : 3. The ratio of HCl : oxalic acid is a little less than 1 : 2.

in equivalent proportions with egg albumin. The curve for H_3PO_4 is the highest curve and if we compare the values for H_3PO_4 with those for HCl (or H_2SO_4) we notice that for each pH the ordinate for H_3PO_4 is as nearly three times as high as that for HCl as the accuracy of our experiments permits. This means that under the conditions of our experiments phosphoric acid combines with albumin in molecular proportions and that the anion of albumin phosphate is the monovalent anion H_2PO_4.

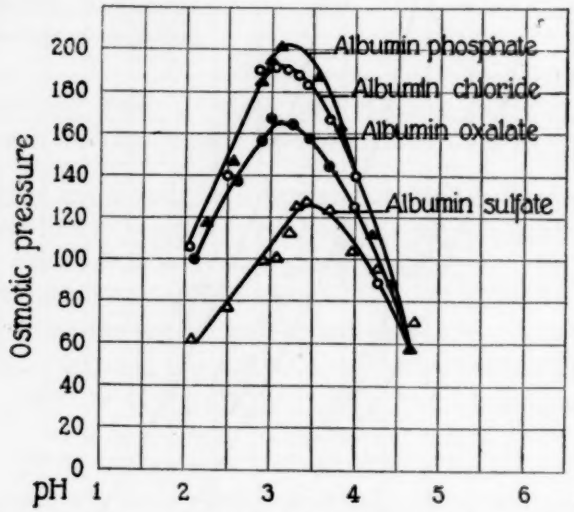

FIG. 4. Osmotic pressure of different albumin-acid salts. The ordinates indicate the osmotic pressure (in mm. of 1 per cent albumin solution); the abscissæ are the pH. All solutions are 1 per cent in regard to isoelectric albumin. The curves for albumin chloride and albumin phosphate are identical.

The values for oxalic acid are for pH above 3.2 not quite twice as high as those for HCl, indicating that for these values of pH oxalic acid combines to a greater extent in molecular and only to a smaller extent in equivalent proportions with albumin.

If egg albumin behaves like gelatin we should expect that the curves of osmotic pressure for albumin phosphate and albumin chloride when plotted as a function of the pH should be identical, since the anion is in both cases monovalent; that the curve for albumin sulfate should

be considerably lower since the anion SO_4 in combination with albumin is bivalent; while the curve for the osmotic pressure of albumin oxalate should be between the curves for albumin chloride and albumin sulfate, but much nearer the chloride than the sulfate curve. Fig.

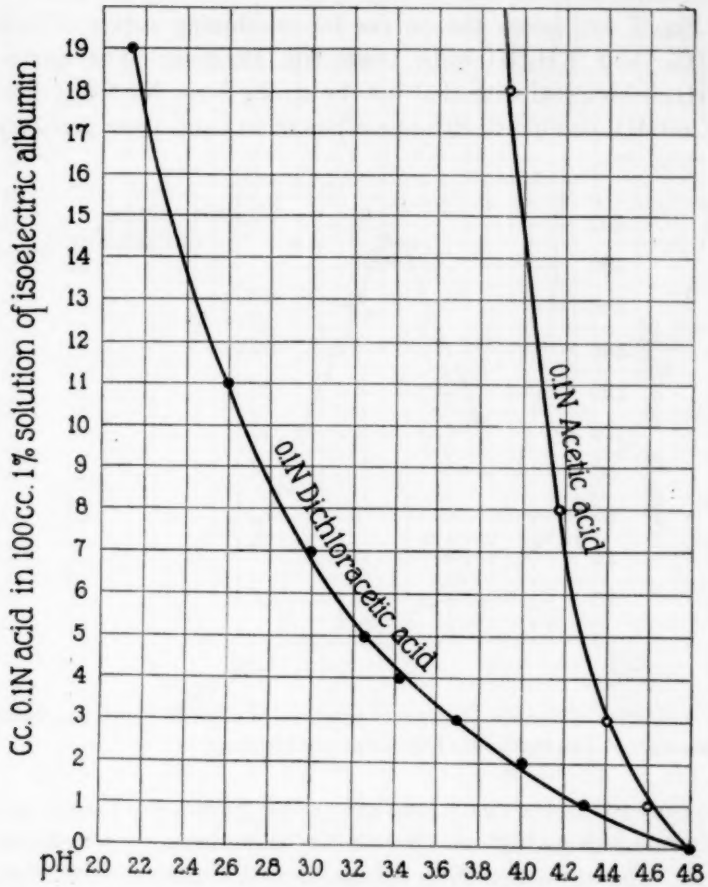

FIG. 5. Curves expressing the cc. of 0.1 N acetic and dichloracetic acids required to bring 1 gm. of isoelectric albumin to different pH.

4 giving the curves of the osmotic pressures of the four albumin salts shows that this expectation is fulfilled.

Fig. 5 gives the curves for the combining ratios of acetic acid and dichloracetic acid with isoelectric albumin, showing the same differ-

ence as the corresponding curves for gelatin in Fig. 1. Fig. 6 gives the curves for the influence of the two acids upon the osmotic pressure of 1 per cent solutions of originally isoelectric albumin. The two curves are identical and are also identical with those of albumin chloride and albumin phosphate in Fig. 4, thus confirming our theory.

In Fig. 7 are given the curves for combining ratios of NaOH, $Ca(OH)_2$, and NH_4OH with isoelectric albumin. The curve for $Ca(OH)_2$ is identical with that for the strong base NaOH, indicating that $Ca(OH)_2$ combines with egg albumin in equivalent proportions.

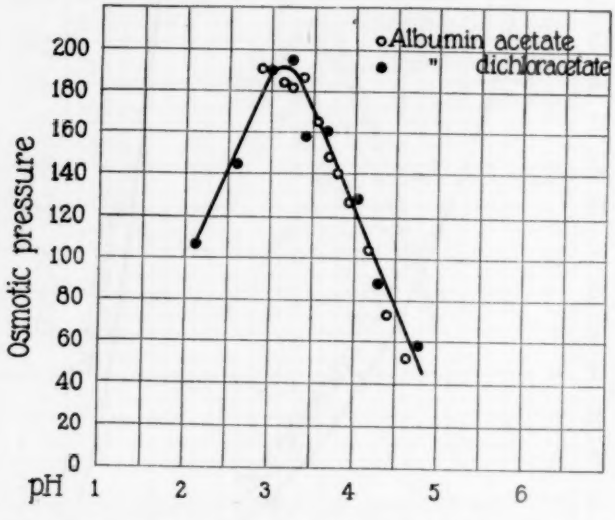

FIG. 6. Curves of osmotic pressure of solutions of albumin acetate and albumin dichloracetate. The curves for both acids are identical.

Fig. 8 shows that the curve for the osmotic pressure of Ca albuminate is only one-half as high as that of Na albuminate as was to be expected. The curve for NH_4 albuminate is identical with that for Na albuminate, which was to be expected since the NH_4 is monovalent.

The results with albumin are therefore identical with those obtained in the case of the corresponding gelatin salts. The result that gelatin phosphate and albumin phosphate behave like gelatin chloride and albumin chloride may be considered as a crucial test against the colloidal conception of ion effects on proteins and in favor of a purely chemical theory.

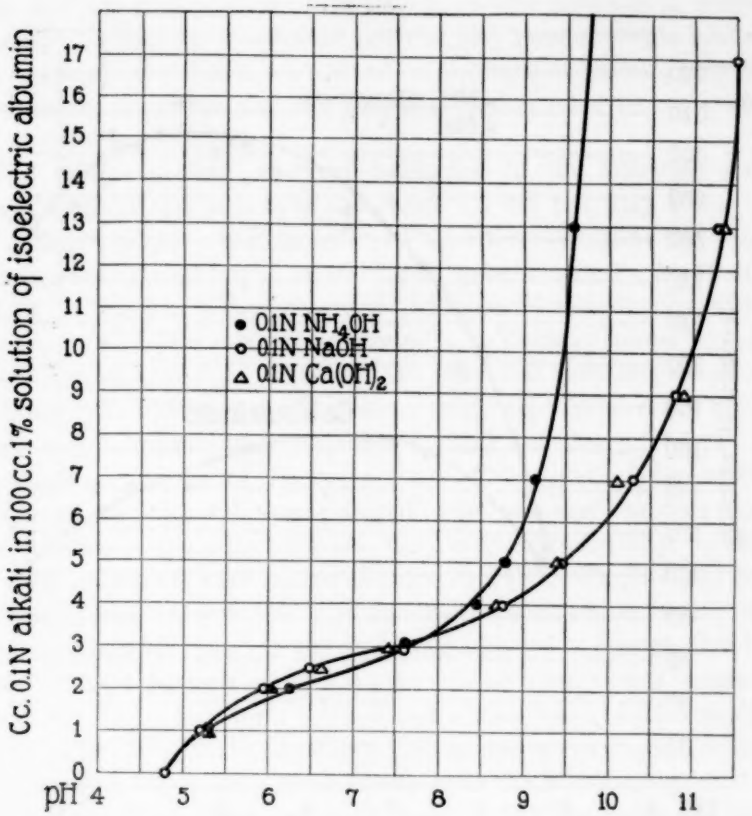

FIG. 7. Curves representing the number of cc. of 0.1 N NH_4OH, NaOH, and $Ca(OH)_2$ required to bring 1 gm. of isoelectric, crystalline egg albumin to different pH. The curves for NaOH and $Ca(OH)_2$ are identical.

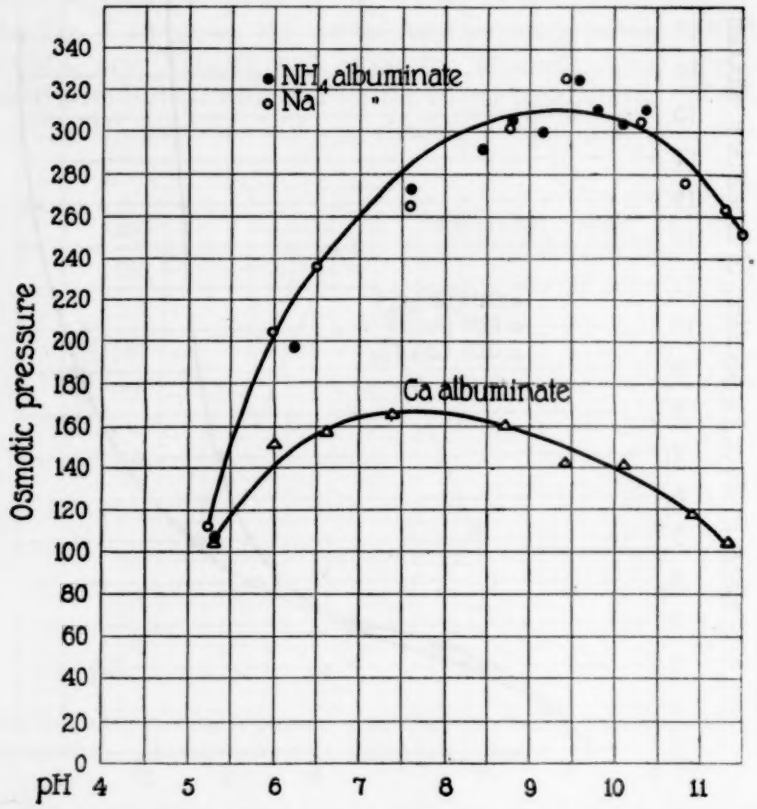

FIG. 8. Curves of the osmotic pressure of NH_4, Na, and Ca albuminate at different pH. The curves for NH_4 and Na albuminate are practically identical.

IV. Combining Ratios of Acids and Bases with Gelatin and the Viscosity of Gelatin Salts.

Since Pauli's ion series was based primarily on the influence of acids or their anions on the viscosity of protein solutions it seemed necessary to find out whether or not viscosity measurements confirm the conclusions at which we arrived on the basis of osmotic pressure experiments on gelatin and egg albumin. A few remarks concerning our method are required.

The gelatin is first rendered isoelectric in the following way. 25 gm. of powdered gelatin of pH about 7.0 are put into 1 liter of M/128 acetic acid for 30 minutes at 10°C., after which time the acetic acid is renewed and left in contact with gelatin again for 30 minutes at 10°C. The acid is then decanted and replaced with distilled water of about 5°C. The mixture is filtered in a Buchner funnel through muslin, employing slight suction. The gelatin is then washed about six times with 100 cc. of distilled water of 5° each, and is made into a 5 per cent solution which serves as a stock solution. The pH of this solution is about 4.7, or, in other words, the gelatin is isoelectric.

Some of the stock solution is heated to 45° and made up to a 2 per cent solution in quantity sufficient for a day's experiments. This 2 per cent solution is kept during the day at 24°C. To 50 cc. of this solution is added the desired acid or alkali in sufficient quantity and then the volume is raised to 100 cc. by the addition of enough distilled water. This 1 per cent solution is then rapidly brought to a temperature of 45°, kept there for 1 minute, and is then rapidly cooled to 24°. The solution is stirred constantly during the heating and cooling. The viscosity is measured immediately after the solution is cooled to 24°C. The measurements were all made at 24°C. by using the time of outflow through a viscometer. The time of outflow of distilled water through an Ostwald viscometer at 24°C. was exactly 1 minute. Each measurement of viscosity was repeated with the same gelatin solution and the beginning and the end of a series consisted in the measurement of viscosity of isoelectric gelatin. These latter measurements agreed in all experiments within 1 second varying only between 80 and 81 seconds, thus guaranteeing the reproducible character of the experiments.

The results can be given briefly. Fig. 9 gives the curves for the cc. of 0.1 N HNO_3, H_2SO_4, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric gelatin to different pH. The curve shows again that for each pH the number of cc. of 0.1 N acid required are for HNO_3, H_2SO_4, oxalic, and phosphoric acids approximately as 1:1:2:3.

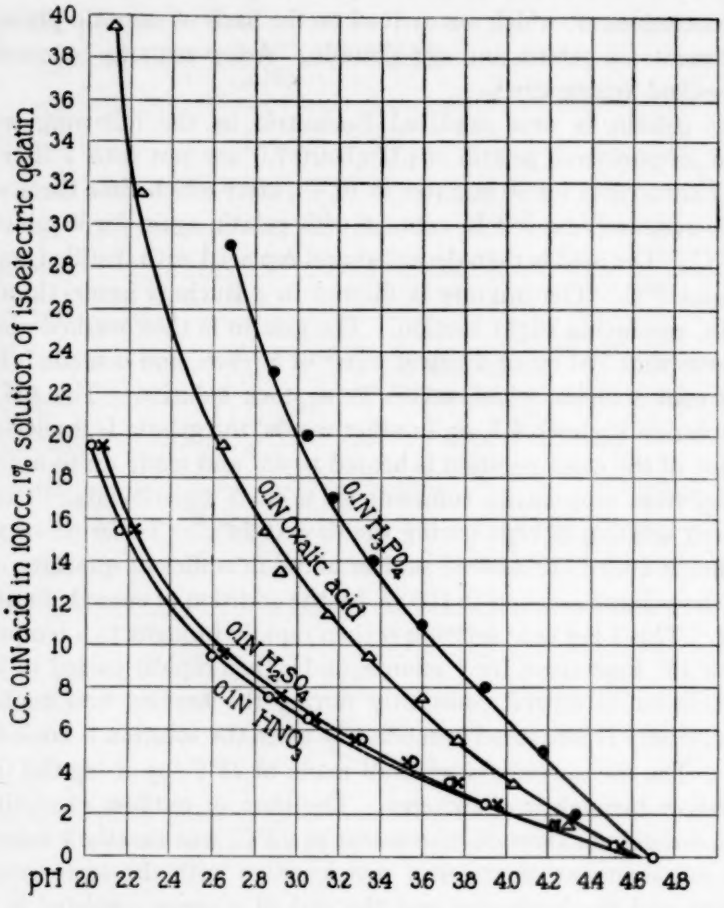

FIG. 9. Curves for the number of cc. of 0.1 N HNO_3, H_2SO_4, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric gelatin to different pH (in 100 cc. of solution). Curves similar to those for egg albumin (Fig. 3). For the same pH the ratio of HNO_3, H_2SO_4, oxalic, and phosphoric acids required is approximately as 1: 1: 2: 3.

Fig. 10 gives the curves for the viscosity of 1 per cent solutions of gelatin chloride, sulfate, oxalate, and phosphate. The abscissæ are the pH, the ordinates the ratio of the time of outflow of the gelatin solutions divided by the time of outflow of pure water. For the sake

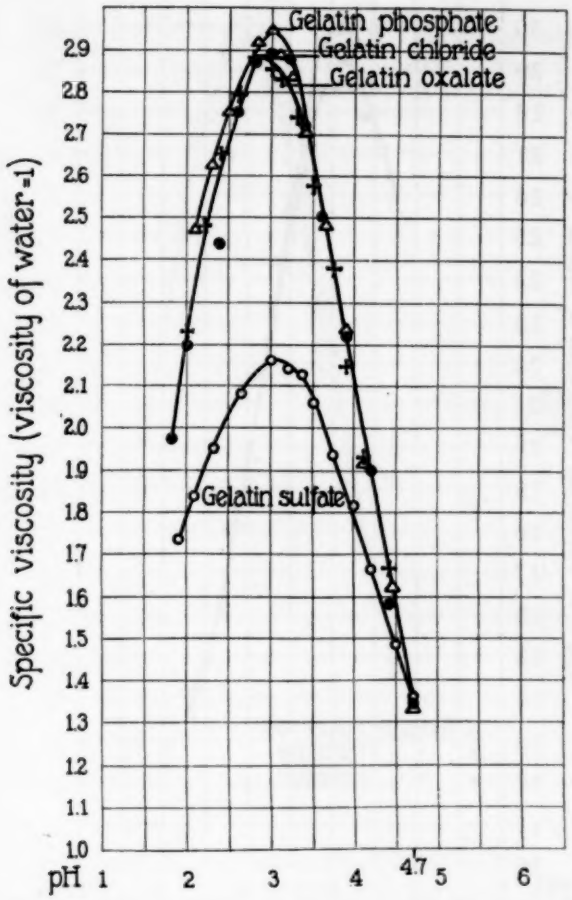

FIG. 10. The curves of specific viscosity of 1 per cent solution of originally isoelectric gelatin brought to different pH by the acids mentioned in legend of Fig. 9 except that HCl is used for HNO_3. The curves of viscosity for gelatin chloride, phosphate, and oxalate are practically identical. Specific viscosity given as time of outflow of gelatin solution divided by time of outflow of water through viscometer at 24°C.

of brevity we will call this quotient the specific viscosity of the gelatin solution. The curves for the four acids all rise steeply from the isoelectric point with increasing hydrogen ion concentration until they reach a maximum at pH about 3.0. The curves then drop again.

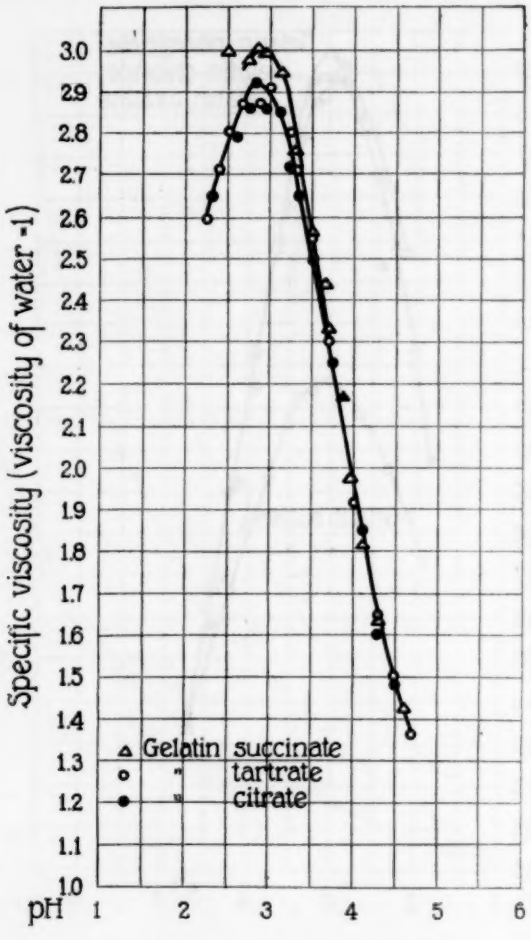

FIG. 11. Curves of specific viscosity of gelatin succinate, tartrate, and citrate. The curves are practically identical with those for the viscosity of gelatin chloride and phosphate.

The curves for the three acids, gelatin chloride, oxalate, and phosphate, are practically identical while the curve for gelatin sulfate is considerably lower.

Fig. 11 gives the curves for the viscosity of gelatin citrate, tartrate, and succinate. The three curves are practically identical and also identical with the curves for gelatin chloride and gelatin phosphate in Fig. 10.

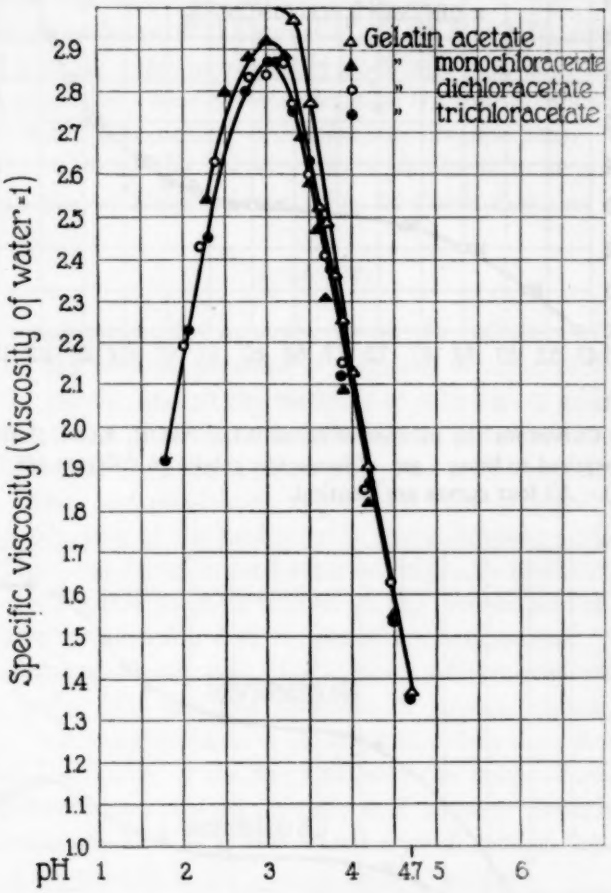

FIG. 12. Curves of specific viscosity of gelatin acetate, mono-, di-, and trichloracetate. Curves identical with those for gelatin chloride and phosphate.

Fig. 12 gives the curves for the viscosity of 1 per cent solutions of originally isoelectric gelatin to which acetic and mono-, di-, and trichloracetic acids have been added. The curves are again identical

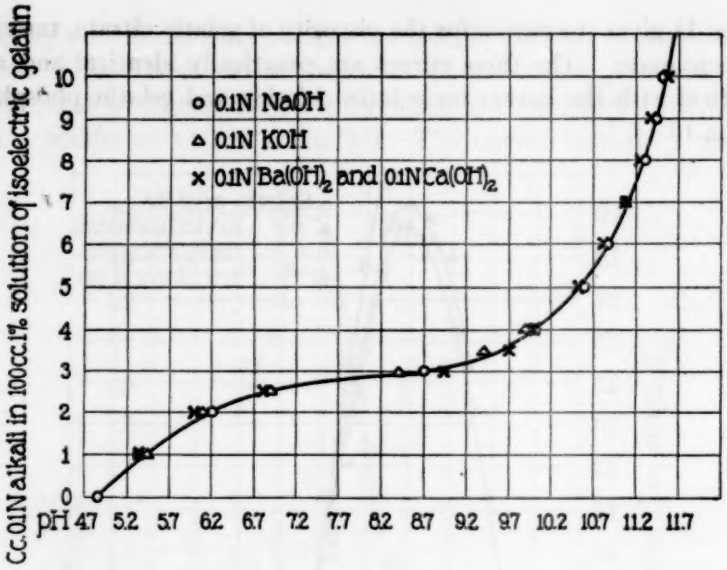

FIG. 13. Curves for the number of cc. of 0.1 N NaOH, KOH, $Ba(OH)_2$, and $Ca(OH)_2$ required to bring 1 gm. of isoelectric gelatin to different pH (in 100 cc. of solution). All four curves are identical.

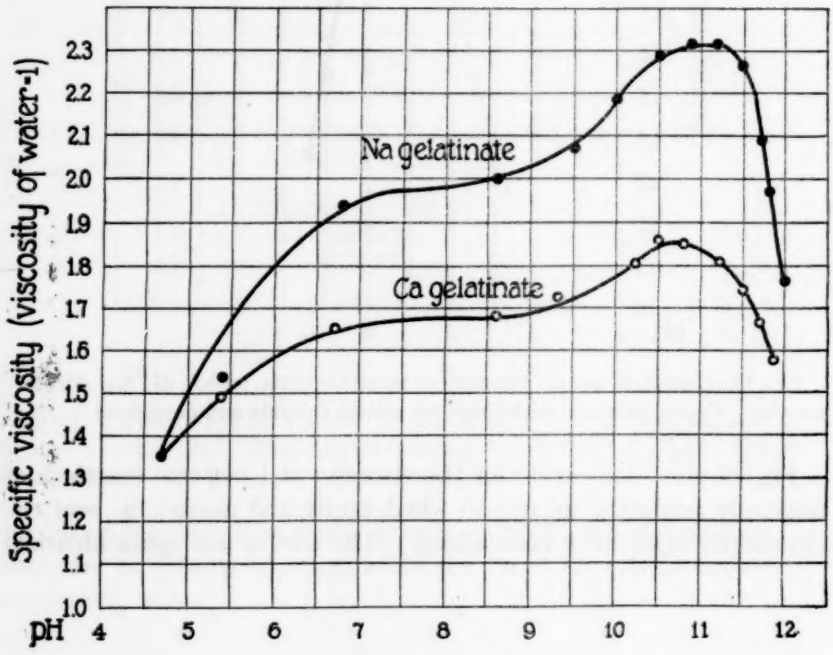

FIG. 14. Curves for specific viscosity of Na and Ca gelatinate for different pH.

with those for gelatin chloride, phosphate, etc. These curves are interesting in connection with Pauli's statement that trichloracetic acid causes the same low values of viscosity as sulfuric acid. This was the case neither in our experiments on the osmotic pressure nor in those on the viscosity of gelatin solutions.

Fig. 13 gives the cc. of 0.1 N NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$ required to bring 1 gm. of originally isoelectric gelatin in 100 cc. solution to a given pH. One curve suffices for the four alkalies thus proving that Ca and Ba combine with gelatin as bivalent ions. We should expect the curve for the viscosity of Ca gelatinate to be considerably lower than that of Na gelatinate. Fig. 14 shows that this is true.

SUMMARY.

1. This paper contains experiments on the influence of acids and alkalies on the osmotic pressure of solutions of crystalline egg albumin and of gelatin, and on the viscosity of solutions of gelatin.

2. It was found in all cases that there is no difference in the effects of HCl, HBr, HNO_3, acetic, mono-, di-, and trichloracetic, succinic, tartaric, citric, and phosphoric acids upon these physical properties when the solutions of the protein with these different acids have the same pH and the same concentration of originally isoelectric protein.

3. It was possible to show that in all the protein-acid salts named the anion in combination with the protein is monovalent.

4. The strong dibasic acid H_2SO_4 forms protein-acid salts with a divalent anion SO_4 and the solutions of protein sulfate have an osmotic pressure and a viscosity of only half or less than that of a protein chloride solution of the same pH and the same concentration of originally isoelectric protein. Oxalic acid behaves essentially like a weak dibasic acid though it seems that a small part of the acid combines with the protein in the form of divalent anions.

5. It was found that the osmotic pressure and viscosity of solutions of Li, Na, K, and NH_4 salts of a protein are the same at the same pH and the same concentration of originally isoelectric protein.

6. $Ca(OH)_2$ and $Ba(OH)_2$ form salts with proteins in which the cation is divalent and the osmotic pressure and viscosity of solutions of these two metal proteinates are only one-half or less than half of

that of Na proteinate of the same pH and the same concentration of originally isoelectric gelatin.

7. These results exclude the possibility of expressing the effect of different acids and alkalies on the osmotic pressure of solutions of gelatin and egg albumin and on the viscosity of solutions of gelatin in the form of ion series. The different results of former workers were probably chiefly due to the fact that the effects of acids and alkalies on these proteins were compared for the same quantity of acid and alkali instead of for the same pH.

THE RECOVERY OF TRANSMISSIVITY IN PASSIVE IRON WIRES AS A MODEL OF RECOVERY PROCESSES IN IRRITABLE LIVING SYSTEMS.

PART I.

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I. INTRODUCTORY.

A striking feature in the phenomena of activation in passive iron wires immersed in solutions of nitric acid is that in acid above a certain critical concentration the reaction is a *temporary* one, followed immediately by an automatic return of the metal to the passive state.[1] Activation initiated at any region of such a wire is thus transmitted rapidly along its whole length in a wave-like manner, each region as it becomes active activating the region next adjoining (by means of the local electric circuit between the passive and active areas) and immediately becoming itself again passive. In this tendency to revert promptly to the chemically inactive or passive state after activation the passive metal resembles an irritable and conducting living element such as a nerve fiber or muscle cell. In order to maintain activity in the living system, constant repetition of stimulation is necessary; and similarly chemical activity in a passive wire immersed in a sufficiently strong solution of nitric acid (55 or more volumes per cent of HNO_3, specific gravity 1.42) is an automatically self-limiting process which can be maintained only by repeated contact of the activating metal; *e.g.*, zinc. Even under such conditions only a partial and irregular activity is possible, which is confined to the immediate neighborhood of the contact; *i.e.*, it fails to be transmitted through more than a short distance. This behavior is highly

[1] For a fuller description of this phenomenon *cf.* Lillie, R. S., *Science*, 1918, xlviii. 51.

characteristic; after a wire has been activated in the usual manner and has reverted to the passive state it is found impossible to reactivate it as a whole by a single contact until a certain interval has elapsed, the duration of which varies with the concentration of acid and with the temperature in a manner to be described later. At first, touching with zinc causes only a brief local reaction which is confined to an area of 1 or 2 cm. from the contact; a second trial made somewhat later gives a reaction which spreads more rapidly and through a greater distance; and with further successive trials the distance through which the activation wave travels, as well as its speed, increases by degrees until eventually rapid transmission through an indefinite distance becomes again possible.

This failure of complete activation and transmission for a certain period succeeding the passage of an activation wave may be compared with the similarly inexcitable and non-conductive interval or "refractory period" of irritable living systems. It is well known that a decline or disappearance of irritability and conductivity during a certain interval succeeding the response to stimulation is a constant feature of the excitation process in all irritable tissues.[2] The duration of this interval varies with the nature of the tissue; the most notable correlation is that it is brief in tissues with rapid rate of response (*i.e.* brief chronaxie), such as voluntary muscle and nerve, and relatively prolonged in slowly responding tissues like the heart or involuntary muscle. In certain cases, as in the photoreceptors of mollusks, the period of diminished sensitivity following the response may last for several minutes;[3] such a phenomenon suggests a fatigue effect, and there are various significant resemblances between the refractory period and cases of brief or evanescent fatigue; thus the period is lengthened by conditions which delay recovery from fatigue, such as lack of oxygen,[4] and also by repeated stimulation, as shown in the

[2] The relation of the refractory period to the excitation process in general is well discussed in the Croonian lecture of Lucas, K., *Proc. Roy. Soc. London, Series B*, 1912, lxxxv, 495.

[3] For a description of this phenomenon *cf.* Hecht, S., *J. Gen. Physiol.*, 1918–19, i, 545.

[4] Verworn, M., Allgemeine Physiologie, Jena, 4th edition, 1903, 559. Fröhlich, F. W., *Z. Physiol.*, 1904, iii, 468.

prolonged "compensatory pause" following an "extra-contraction" in heart muscle.

It is generally assumed that the refractory phase corresponds essentially to the period required for recovery or recuperation. The act of stimulation appears to involve the more or less complete breakdown or removal of some material or structure which is essential to stimulation; the refractory interval represents a period of reconstruction or restoration. When this process of repair is rapid the refractory period is brief, and *vice versa*. In any single tissue the rate of this reconstruction exhibits a general parallelism with the rate of the structural or chemical breakdown associated with stimulation; hence rapidly responding tissues have as a rule brief refractory periods. This parallelism can, however, be artificially disturbed, as in Tait's experiments, by drugs like protoveratrine and yohimbine which abnormally prolong the refractory period. In such cases an abnormal prolongation of the descending or return phase of the bioelectric variation is also observed.[5] There appears thus to be a correlation between the length of the refractory period and the duration of the bioelectric variation, especially of the return phase of the latter. The relation, however, is not simple, for it is certain that in some cases, *e.g.* heart muscle, the whole period of diminished excitability (including both the "absolute" and the "relative" refractory phases) may greatly outlast the electrical variation.[6] This is also true of the metallic model under consideration in the present paper. It is thus not sufficient to regard the entire refractory phase as corresponding to the time required to restore the normal or "resting" semipermeability and polarizability of the altered plasma membranes of the irritable elements. Recovery of semipermeability is no doubt necessary to a recovery of irritability, since electrical stimulation requires polarizability in the membranes;[7] but some further change appears also to be essential. This question will be discussed later, after the processes in the metallic model

[5] Tait, J., *Quart. J. Exp. Physiol.*, 1910, iii, 221.

[6] Trendelenburg, W., *Arch. ges. Physiol.*, 1912, cxliv, 39. The case of nerve, with a brief refractory period, is similar (*cf.* Adrian, E. D., *J. Physiol.*, 1914, xlviii, 453).

[7] The inference from the work of Nernst, Lapicque, Lucas, Hill, and others on electrical stimulation.

have been considered. It seems probable that the presence of a brief "absolute," preceding a longer "relative," refractory phase is an index of the existence of two distinct stages or processes in the reconstitution of the plasma membrane, the first stage preceding and the second succeeding the recovery of semipermeability. This view, however, accords imperfectly with Tait's special hypothesis that the "absolute" refractory period corresponds to the period of upstroke of the bioelectric variation, and the "relative" period to the return phase. In the metallic model the change of potential accompanying repassivation, *i.e.* the return phase of the whole electrical variation associated with the local reaction, is a definite index of the reformation of the passivating surface film of oxygen compound. This process occurs rapidly; but it is not until the lapse of a considerable time (usually some minutes) after its completion that the condition of the film becomes such as to permit again of ready and complete transmission. Evidently some further change in the newly restored film is necessary for a return of its original properties; and it seems probable that in the living system closely comparable conditions may obtain. It is certain that the relative refractory period greatly outlasts the return phase of the bioelectric variation in many cases.

In the living irritable element there is evidence that stimulation is associated with a temporary alteration or breakdown of the protoplasmic surface film or plasma membrane, involving a general increase of permeability.[8] Stimulation follows any sufficiently rapid and extensive change in the electrical polarization of the semipermeable protoplasmic surface, or any sufficient local mechanical or chemical alteration; such a change, even though purely physical in itself, initiates a chemical disturbance, associated with a variation of potential, which sweeps over the cell surface, and apparently consists essentially in a wave of alternate dissolution and reconstruction of the surface film. This "propagated disturbance"[9] is the condition for the release of the chemical and other activities in the cell interior which constitute

[8] For a brief summary of this evidence *cf.* Lillie, R. S., *Am. J. Physiol.*, 1915, xxxvii, 356. Further references there given.

[9] Keith Lucas' term, to distinguish the transmitted effect from the local or initiatory effect produced by the stimulating agent. For the experimental basis of this distinction *cf.* Adrian, E. D., and Lucas, K., *J. Physiol.*, 1912, xliv, 68.

the response to stimulation. Normally in a highly irritable element, such as a muscle cell or nerve fiber, the whole cell surface is thus involved, hence the "all or none" character of the response; but the passage of the excitation wave, and with it the local response, can be interfered with or prevented by various artificial conditions (narcosis, electrotonus, local chemical or mechanical conditions). There are many purely physiological indications that the transmission of excitation from one region of the conducting element to the next adjoining is due to the local bioelectric variation resulting from this surface alteration.[8] The reference of the bioelectric variation to a local change in the physical and chemical character of the cell surface, which apparently acts like an electrode of variable chemical composition and potential, is consistent with what we know of the conditions in various inorganic systems exhibiting an analogous type of behavior; examples of such systems are mercury in hydrogen peroxide,[10] chromium and other electrodes exhibiting rhythmical changes of potential,[11] and especially metals in the passive state, more particularly iron.[12]

Activation and transmission in passive iron are due to alterations in the continuous or impermeable[13] surface film of oxidation product covering the surface of the metal. On account of the extreme thinness of this film, the surface of the passive metal, in its property as an electrode, is highly variable and sensitive to mechanical or other disturbance. The susceptibility of a passive iron wire to activation by mechanical and chemical agents and by the electric current (when the metal is made cathode) is thus readily explained. Interruption of the film produces a local circuit, and electrolyses at the electrode areas of this circuit—reduction at the cathodal and reoxidation at the anodal areas—are responsible both for the rapid extension of the active area (transmission) and for the automatic return to the passive

[10] Bredig, G., and Weinmayr, J., *Z. physik. Chem.*, 1903, xlii, 601. Bredig, G., and Wilke, E., *Biochem. Z.*, 1908, xi, 67. Von Antropoff, A., *Z. physik. Chem.*, 1908, lxii, 513.

[11] Ostwald, W., *Z. physik. Chem.*, 1900, xxxv, 33, 204.

[12] The general article by Bennett and Burnham (Bennett, C. W., and Burnham, W. S., *J. phys. Chem.*, 1917, xxi, 107) gives a full account of the phenomena of passivity in metals and an exhaustive literature list.

[13] *I. e.* impermeable to acid, hence its protective influence.

state. The resemblances between the phenomena of activation and transmission in the passive iron model and in living protoplasm are thus to be referred to the presence, in both systems, of surface films which readily undergo chemical and structural alteration, these alterations producing changes in the electromotor properties of the surface. Such local alterations have further chemical effects, since they produce local electric circuits which furnish the conditions for electrolysis, and these chemical effects are often extensive because of their automatic tendency to spread wherever a continuous and uniform surface film is present.

The general biological significance of these properties of surface films, especially in relation to the film-pervaded or emulsion-like structure of living protoplasm—emulsions being systems whose properties are determined by the presence of surface films of soap or other material—will be partly evident from the foregoing, but will not be considered in detail in the present paper.[14] It is essential to note, however, that recovery of irritability after stimulation depends (on the foregoing theory) upon the restoration of the altered protoplasmic surface film to its previous condition. It seems most likely that an essentially new film is formed after each stimulation; this process of reconstruction probably involves a specific chemical resynthesis, in addition to a purely physical process of redistribution or rearrangement of surface active compounds. The importance of the chemical factor is indicated both by the dependence of recovery on oxygen and by its high temperature coefficient.[15] In the passive iron model the factors in the recovery of transmissivity are simpler than in protoplasm; but repassivation is known to depend upon the formation of a new surface film of oxidation product; and immediately after the redeposition of this film its properties are different from those which it attains later. We can in fact clearly distinguish two distinct processes in the recovery of passive iron; one the redeposition of the film, and the other the progressive alteration of the newly deposited film until the final state of complete transmissivity is attained. The first process

[14] For an account of various biological analogies in the behavior of the surface film of passive iron, *cf.* Lillie, R. S., *Science*, 1919, l, 259, 416.

[15] Bazett, H. C., *J. Physiol.*, 1907–08, xxxvi, 414. Adrian.[6]

is associated with a change in the electrical potential of the metallic surface, from that characteristic of active iron (in contact with acid) to that of the passive or oxide-covered surface. This variation of potential is a direct and readily observable index of the return to the passive state; it occurs simultaneously with the decline and cessation of the reaction (effervescence, etc.) and lasts for only a short time, *e.g.* a fraction of a second; the succeeding phase of gradually returning transmissivity is much more prolonged. A somewhat similar division of the recovery process into two stages can also be distinguished in living tissues, especially those with relatively long refractory periods, *e.g.* heart muscle, as already indicated. These resemblances justify the belief that a study of the conditions of recovery in the metallic system may throw light on the general nature of the recovery process in living protoplasm.

II. Phenomena of Automatic Repassivation in Iron Wires.

The following account is based on experiments with iron wires of standard composition and properties, known as "music steel wires," manufactured by the Spencer Wire Company of Worcester, Massachusetts. Wires of 1 to 1.2 mm. diameter were used (Nos. 20 and 25). The metal is bright, elastic, and highly tempered. Its behavior in nitric acid of different concentrations is definite and regular, within certain well defined limits of variation to be described below. It is important in experiments of this kind to use metal of uniform quality, for ordinary specimens of iron (*e.g.* "black iron" sheeting, soft iron wire, wire nails, etc.) are often highly variable and capricious in their manner of reaction with strong nitric acid.

I have already described briefly the appearances accompanying the activation of these wires in different dilutions of pure nitric acid.[1] Before use, the freshly cut wires are allowed to react for 15 seconds or so with dilute acid (specific gravity 1.2); they are then rinsed in water, wiped clean with a coarse cloth, and passivated by immersion in strong HNO_3 (specific gravity 1.42). The passive wires are steel bright and undergo no change if left undisturbed in dilute nitric acid (from specific gravity 1.2 up). When they are activated in dilute acid, *e.g.* by touching with zinc or scraping with glass, there is an

immediate darkening of the bright surface (from formation of oxide), followed by active effervescence; both of these changes then spread rapidly along the whole length of the wire. A completely transmissive wire thus reacts as a whole, in a manner which in acid of a definite concentration and temperature is constant and independent of the mode of activation. The reaction continues for an indefinite time in acid below a certain concentration; this critical concentration is about 50 volumes per cent of 1.42 HNO_3 (equal to acid of 1.2 specific gravity or about 7.5 N by volume); in all stronger solutions the reaction soon comes automatically to rest and passivity is regained. In such cases the duration of the local reaction varies in a characteristic manner with the concentration of HNO_3; *e.g.*, in acid of 85 volumes per cent (about 12 N by volume) effervescence lasts (at 20°) for only a small fraction of a second, while in 55 per cent HNO_3 it continues for several seconds, at first uniformly and later declining and finally ceasing somewhat abruptly, usually after 6 to 8 seconds. During the reaction a dark brown oxide is deposited on the surface of the metal; the thickness of this coating depends on the duration of the reaction; hence immediately after spontaneous repassivation in 55 per cent acid a wire is almost black in color, while in 80 per cent acid it is only slightly bronzed. This deposit gradually dissolves away if the metal is left in the acid; it is loosely adherent and is entirely distinct from the thin, resistant, and invisible surface film which imparts passivity. The recently formed oxide-covered region furnishes a convenient indicator of the distance traveled by the activation wave in cases of partial transmission, since its boundary against less recently activated regions is always sharply defined.

On activation the potential of the metal changes suddenly from that characteristic of passive to that of active iron; in nitric acid of the above concentrations the active metal is more negative (zinc-like or anodal) by 0.7 to 0.8 volt. Hence the duration and certain other features of the reaction can be observed conveniently by the use of a voltmeter or string galvanometer. When two iron wires, bent into an appropriate shape and connected through keys to a voltmeter (preferably one with a scale of 1 to 2 volts and a central zero), are passivated by dipping into 1.42 HNO_3, and are then placed side by side a short distance apart in a flat dish containing 55 per cent acid, on

activating one of the wires the needle of the instrument shows at once a rapid initial excursion of about 0.7 volt. During the continuation of the reaction the potential exhibits irregular rhythmical fluctuations about this point; later, as the reaction begins to decline, it decreases by degrees, finally swinging back rather abruptly to zero and a little beyond as the reaction ceases. It is characteristic that immediately after repassivation the wire is always slightly more positive than before, by a potential of 0.01 to 0.02 volt, and remains in this condition for some time; by degrees, especially if the acid is stirred, the wire resumes its original potential. The rhythmic variation of potential is an especially interesting peculiarity from the biological point of view, since rhythmicity of a similar kind is frequent in irritable living tissues, as well as in other film-covered inorganic systems, such as mercury in hydrogen peroxide. It indicates a tendency in the early stages of the reaction to an alternating formation and disruption of the passivating film; later the process of formation predominates and the film becomes continuous over the whole surface; the wire is then passive. The "after-positivity" has an analogy to the so called "positive after-variation" observed under certain conditions in nerves after stimulation;[16] from the most general point of view it is to be regarded as a polarization effect of the kind found at all polarizable electrodes.

Time Relations of Recovery of Transmissivity.—The behavior of a wire in nitric acid of 60 per cent concentration will first be described for illustration. When such a wire, after having been left undisturbed in this solution for some time (*e.g.* an hour), is touched at one end with zinc a wave of activation sweeps rapidly (at some hundred centimeters a second) over its whole length; after 1 or 2 seconds the reaction ceases and the metal becomes again passive. For some minutes after repassivation the wire is found to be incapable of transmitting activation for more than a limited distance. At the very first, touching with zinc produces only a slight local reaction; when touched 30 seconds later the local effervescence and darkening caused by the zinc are somewhat more pronounced, and the reaction spreads slowly for 2 or 3 cm. from the contact; after 1 minute there is somewhat more rapid transmission through 4 or 5 cm.; after 2

[16] Tigerstedt, C., and Donner, S., *Skand. Arch. Physiol.*, 1913, xxx, 309.

minutes the speed is still greater and the distance has increased to 10 or 15 cm.; and after 3 minutes the wave usually travels rapidly (though less so than before the original activation) over the whole length as before. Recovery is thus a gradual process whose course can be traced by measuring the distance which an activation wave travels at successive intervals after repassivation. In the experiments about to be described the relation between the concentration of acid and the rate of recovery has been determined in this manner for solutions of nitric acid of different concentration.

The procedure has been as follows. Straight lengths of wire 20 cm. long are bent at one end into a hook shape (to facilitate handling, which is done with glass hooks and rods) and treated as already described so as to secure a uniform surface; they are then passivated by immersion in strong nitric acid (Baker's "Analyzed," specific gravity 1.42), where they may remain for an indefinite time before using. The same wires may be used over and over again in different experiments. In each experiment with a given solution several such wires, *e.g.* ten, are placed side by side in a flat-bottomed rectangular glass dish (23 by 14 cm.) containing the acid; when all the wires are in position they are activated simultaneously (or nearly so) by touching at one end with a bar of zinc; an activation wave then sweeps rapidly over each wire, which receives at the same time a coating of the brown oxide. The wires are now again passive, but at first transmit activation imperfectly, as just described. The distance traveled by the activation wave after a definite time interval of recovery is now determined by touching the ends of the wires with zinc, one by one, in succession from left to right at regular intervals. For instance, in 65 per cent acid the successive wires are activated at $\frac{1}{2}$ minute intervals; a series is thus obtained showing the distances traveled after periods of recovery lasting respectively $\frac{1}{2}$ minute, 1 minute, $1\frac{1}{2}$ minutes, 2 minutes, etc. The distances can readily be measured with a millimeter rule; the limit of each freshly activated area is distinctly marked by the dark shade of the more recently deposited oxide; this boundary remains visible for some time after the reaction, so that if desired the measurements can be made at leisure at the conclusion of a series of experiments.

The rate of recovery, as measured by the gain per unit of time in the traveling power of the wave, is at first gradual and then more rapid. Transmission for the full distance of 20 cm. is equivalent to transmission for an indefinite distance; it is in fact unusual for a wave that has traveled 16 cm. (at 20°) to fail to travel the whole length of any wire. Transmission for 20 cm. thus signifies full or complete transmissivity. If we define transmissivity in numerical terms as the distance in centimeters which the wave is capable of traveling along a homogeneous wire before spontaneous extinction, its values range from unity or less to infinity, the latter value signifying transmission without progressive decline in traveling power. This condition corresponds to an "all or none" behavior, or conduction without decrement, in a living conducting tissue like nerve; partial transmission is equivalent to conduction with a decrement; the numerical measure of this decrement may be defined as the reciprocal of the distance traveled before extinction.

The behavior of different wires under otherwise identical conditions is subject to considerable variation, due apparently to accidental differences in the structure of the metal in the different wires. Thus there are occasional exceptions to the rule of an increase in the distance of transmission on passing from one member of a series to the next. Table I gives the results of seventeen separate series of determinations with wires immersed in 65 volumes per cent of 1.42 acid (= about 9 N by volume). These series were all carried out on a single day, using two sets of ten wires each; these were used alternately, a determination with the one set being made while the other was undergoing repassivation in strong acid. There are thus eight series of observations with the one group and nine with the other; the wires were placed in random order in each series; *i.e.*, no care was taken to treat each wire individually as in the experiments to be described later. The figures give the distances in centimeters traveled by the activation wave along the wire when the latter is touched at one end with zinc after the intervals (since the previous complete activation) given at the head of the column. Each horizontal line gives the observations with a single series. The temperature of the acid was constant between 20 and 21°.

It will be observed that complete recovery occupies about 4 minutes in this solution; in three series (four wires) transmission was complete after $3\frac{3}{4}$ minutes; twelve series (eighteen wires) required 4 minutes, and two $4\frac{1}{4}$ minutes. The average distance of transmission at the

TABLE I.

Concentration of HNO_3, 65 Volumes Per Cent. Temperature 20–21°C.

Series.	Distance traveled. Intervals since previous activation.									
	½ min.	1 min.	1½ min.	2 min.	2½ min.	3 min.	3½ min.	3¾ min.	4 min.	4¼ min.
	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.
A	2.6	4.0	4.0	7.2	8.4	8.9	15.0		Full.	
B	2.6	4.2	5.1	6.4	7.0	8.3	14.4		"	
C	2.6	2.3	3.9	4.6	7.2	8.8	15.7	Full.		
D	2.1	2.9	4.5	5.3	6.0	7.8	13.7	"		
E	1.9	2.6	3.1	5.0	7.1	8.4	13.6	"		
								(2) 15.1		
F	3.1	3.0	3.6	4.4	5.7	5.9	8.2	12.2	16.0	Full.
G	2.0	3.7	2.9	3.7	7.8	7.5	11.3	13.1	16.1	"
H	2.5	3.9	3.6	4.6	6.1	7.9	8.7	15.0	Full (2).	
I	2.1	2.9	3.9	3.6	6.2	7.4	10.3	14.7	" (2).	
J	1.7	2.4	3.9	4.6	5.7	6.7	10.4	13.6	" (2).	
K	2.5	4.3	3.5	3.7	5.6	6.1	8.3		" (3).	
L	1.5	2.5	3.7	4.4	4.2	8.4	10.5	11.9	" (2).	
M			4.5	4.7	5.4	7.5	10.7	14.0	"	
N	2.0	3.1	2.9	3.7	5.6	7.5	10.6	13.6	" (2).	
O	2.3	2.2	2.8	4.7	5.3	5.9	7.5	10.5	" (2).	
P	1.9	2.3	3.3	4.2	5.8	5.6	8.1	9.3	" (1). 12.2 (1).	
Q	2.2	3.0	3.3	3.0	5.8	5.9	9.8	9.9	Full (2).	
Average..	2.2	3.1	3.7	4.6	6.2	7.3	11.0	12.7	14.8	

end of each interval is given at the foot of the column. The increment (per unit of time) of transmissivity during the whole recovery period of 4 minutes is at first gradual and more rapid later. The transition from a limited to an unlimited transmissivity occupies a comparatively short interval toward the end of the period. This

feature of the behavior has an important general significance, as will be seen below.

The same type of behavior was found in all the solutions used, which included nitric acid of the following concentrations (in volumes per cent of 1.42 HNO_3): 55, 57.5, 60, 65, 70, 75, 80, 85, 90. The time required for complete recovery of transmissivity increases rapidly with increasing concentration of acid. Table II gives the typical recovery times for different solutions at 20°. In the most concentrated solutions, 85 and 90 per cent, especially the latter, the recovery process may exhibit irregularities whose explanation is not entirely clear; and at times transmissivity remains incomplete, even

TABLE II.

Concentrations (1.42 HNO_3).	Time for complete recovery (20°).
vol. per cent	
55.0	30 – 45 sec.
57.5	60 – 70 "
60.0	90 –110 "
65.0	3½– 4 min.
70.0	6 – 7 "
75.0	9 – 10 "
80.0	12 – 13 "
85.0	14 – 16 "
90.0	18 – 20 " (or more).

after hours or days in these solutions. Apparently very strong acid renders the surface film resistant to alteration under certain conditions. Frequently, however, there is complete recovery in 90 per cent or even stronger acid; but the time required is usually 20 minutes or more. In the lower concentrations complete transmissivity always returns within the time indicated in Table II.

In their general character the measurements for the different solutions resemble those given in Table I, so that it is unnecessary to reproduce them in detail; Table III, however, gives the averages obtained for the different solutions from 57.5 per cent on.[17] In these

[17] Exact measurements with 55 per cent acid are difficult to make because the wire at the end of the reaction (which lasts 5 or 6 seconds) is so covered with oxide that the distance traveled by a later activation wave is not distinctly marked. Recovery is more rapid and the whole behavior is less regular than in the stronger solutions.

TABLE III.

Concentration of acid.	Average distances traveled by activation wave in each solution at definite intervals after previous activation.																			
vol. per cent	*sec.*	*cm.*	*sec.*	*cm.*	*sec.*	*cm.*	*sec.*	*cm.*	*sec.*	*cm.*	*sec.*	*cm.*	*sec.*	*cm.*	*sec.*	*cm.*	*sec.*	*cm.*	*sec.*	*cm.*
57.5					10	3.4	20	6.5	30	9.6	40	11.3	50	14.0 (Full transmission in 4 wires out of 19.)						
60.0	*min.*		20 *min.*	5.3	30 *min.*	6.3	40 *min.*	7.0	50 *min.*	7.6	60 *min.*	8.9	70 *min.*	12.6	80 *min.*	12.7	90 *min.*	13.4 (5 full in 19.)	100 *min.*	16 full in 19.
65.0	½	2.2	1	3.1	1½	3.7	2	4.6	2½	6.2	3	7.3	3½	11.0	3¾	12.7 (4 full in 16.)			4	14.8 (3) (12 full in 15.)
70.0	1	1.3	2	1.5	3	2.3	4	3.8	4½	4.2	5	5.9	5½	6.7	6	10.1 (2 full in 14.)	6½	11.3 (5) (7 full in 12.)	7	All full.
75.0	1	2.0	2	2.2	3	3.0	4	3.4	5	4.3	6	5.8	7	7.7	8	10.1	9	10.8 (3) (9 full in 12.)	10	" "
80.0	2	2.0	4	2.7	6	3.6	8	5.1	10	7.3			12	10.3 (3 full in 17.)			13	All full.		
85.0	2	3.1	4	3.1	6	3.5	8	4.6	10	7.4	12	8.6	13	10.4 (5)	14	12.8 (2 full in 14.)	15	12.4 (5) (8 full in 13.)	16	5 full in 6.
90.0	2	2.5	4	2.9	6	3.8	8	4.3	10	5.4	12	6.7	14	9.5			16	11.0 (10) (6 full in 16.)	19	13.9 (2) (4 full in 6.)

experiments the intervals between the successive activations in any series were adjusted to the time period of recovery characteristic of the solution used, as given in Table II. In each series (with a few exceptions) ten wires were used, with the procedure already described. The number of separate determinations for each of the average distances given in Table III varied from twelve to nineteen (in a few cases more), except in those cases where part of the wires had recovered

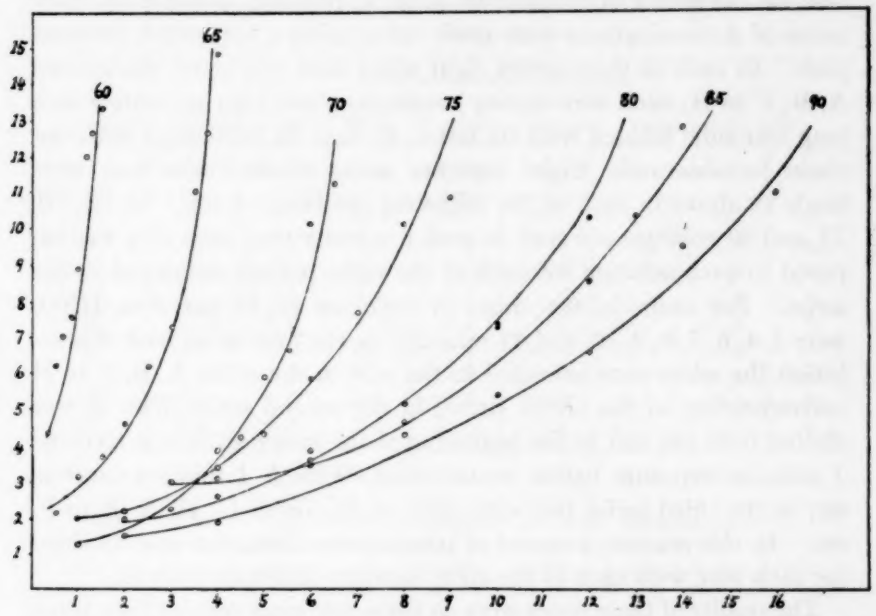

FIG. 1. Distances traveled by activation wave in different solutions of HNO_3 at different intervals after a previous complete activation in the same solution. Ordinates are distances (cm.); abscissæ are times (minutes) elapsed since the previous activation. A single curve represents the behavior in acid of the concentration indicated at the end of the curve (in volumes per cent of HNO_3, sp. gr. 1.42) at 20°. The small circles represent the averages given in Table III.

completely in the time allowed; in these cases the number of determinations is given in brackets. When plotted with distances as ordinates and times as abscissæ the data give curves of an hyperbola-like form, beginning with a horizontal and ending with a vertical course (Fig. 1).

All these determinations were made at room temperature, and the temperature of the acid was taken at the end of each series; in all but seven of the 119 series summarized in the table the temperature was between 19 and 21°; in the others it rose to 21.5 or 22° (in one case 22.5°). The irregular fluctuations in the series have, however, nothing to do with temperature, but are to be referred chiefly to accidental variations in the surface structure of the different wires. In order to obviate this source of irregularity, as far as possible, a number of other series of determinations were made later, using a somewhat different plan. In each of these series eight wires were employed, designated A, B, C to H; each wire during passivation was kept separately in a long test-tube labeled with its letter, so that its individual behavior could be observed. Eight separate series of determinations were made as above in each of the following solutions of acid: 60, 65, 70, 75, and 80 volumes per cent, in such a manner that each wire was exposed to each solution for each of the eight periods employed in the series. For example, the times of exposure to 75 per cent HNO_3 were 2, 4, 6, 7, 8, 9, 10, and 11 minutes; in the first series with this solution the wires were arranged in the acid in the order A, B, C to H corresponding to the above times; in the second series Wire H was shifted from the end to the beginning of the series, so that it received 2 minutes exposure before reactivation, Wire A 4 minutes, and so on; in the third series the wires were in the order G, H, A, B to F, etc. In this manner a record of transmission distances was obtained for each wire with each of the eight recovery intervals allowed.

The results of these series were on the whole more regular than those of the earlier series. The wires used were of the same kind as before, but cut from a different roll (No. 25 thickness). The behavior and the recovery times showed little difference from those of the earlier series (made with No. 20 wire). Care was taken to keep the temperature constant between 19.5 and 20.5°. Table IV gives the complete record of two typical series with 65 and 80 per cent acid respectively.

These results are closely similar to those obtained in the earlier series, and no significant differences of behavior are observable in the individual wires. It is apparent that each reaction means a renewal of the reacting surface, since a thin layer of metal is dissolved each time. The irregularities of behavior are therefore probably due to

variations in the finer grain of the metal, involving irregular local potential differences which influence the local rate of reaction and interfere somewhat with the uniformity of the reaction at different regions of the surface layer. In Fig. 2 the averages for the different solutions of these series are plotted and curves drawn as before. These averages are given in Table V.

TABLE IV.

Wire.	Distance traveled. Intervals since previous activation.							
Series 1. Concentration of HNO_3, 65 volumes per cent. Temperature 20°C.								
	1 min.	1½ min.	2 min.	2½ min.	3 min.	3½ min.	4 min.	4½ min.
	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.
A	4.2	4.8	4.5	5.5	8.1	10.4	11.7	Full.
B	3.2	3.2	4.1	4.0	4.9	8.3	13.3	"
C	3.2	4.4	4.6	5.7	7.5	9.1	12.9	"
D	4.0	5.2	4.4	8.3	7.4	10.2	11.5	"
E	2.3	3.5	4.2	7.1	10.5	10.3	13.5	"
F	2.8	3.9	4.9	5.9	8.4	10.5	15.1	"
G	3.8	2.5	5.2	5.9	9.6	9.9	12.0	"
H	3.1	4.5	5.0	5.5	7.3	11.0	15.5	"
Average.	3.3	4.0	4.6	6.0	8.0	10.0	13.4	
Series 2. Concentration of HNO_3, 80 volumes per cent. Temperature 20°C.								
	2 min.	4 min.	6 min.	8 min.	10 min.	12 min.	13 min.	14 min.
A	1.2	1.5	2.3	3.2	5.2	13.2	16.8	Full.
B	1.5	1.8	2.0	3.4	6.8	9.4	Full.	"
C	1.9	1.9	3.1	3.4	6.4	11.3	18.8 Full.	
D	1.7	2.7	2.7	3.3	4.5	8.9	11.6	Full.
E	1.8	2.5	2.7	3.8	6.1	7.6	14.5	"
F	1.4	2.1	2.4	4.6	6.4	11.2	9.0	"
G	1.3	2.0	3.0	4.5	7.2	9.9	12.0	10.7
H	1.5	1.6	2.6	4.1	5.4	11.2	13.4	Full.
Average.	1.5	2.0	2.6	3.8	6.0	10.3	13.7	

In this arrangement the horizontal lines give the results for the same wire with varying intervals before reactivation. Any single series is to be read along the diagonals from above down, beginning with the first column, in such a way that the letters fall in consecutive order.

TABLE V.

HNO_3	Distance travelled.																	
	Interval since previous activation.																	
per cent	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*	*min.*	*cm.*
60	½	2.5	¾	3.7	1	4.2	1¼	5.5	1½	6.8	1¾	10.3	2	13.0 (2 full in 8.)			2¼	13.1 (3) (5 full in 8.)
65	1	3.3	1½	4.0	2	4.6	2½	6.0			3	8.0	3½	10.0	4	13.4	4½	All full.
70	1	2.3	2	3.0	3	3.8	4	4.8			5	5.9	5½	7.0	6	9.4	6½	6 full in 8.
75	2	2.0	4	3.0	6	4.2	7	5.8			8	7.8	9	10.8	10	13.1 (3) (5 full in 8.)	11	All full.
80	2	1.5	4	2.0	6	2.6	8	3.8			10	6.0	12	10.3	13	13.7 (1 full.)	14	7 full in 8.

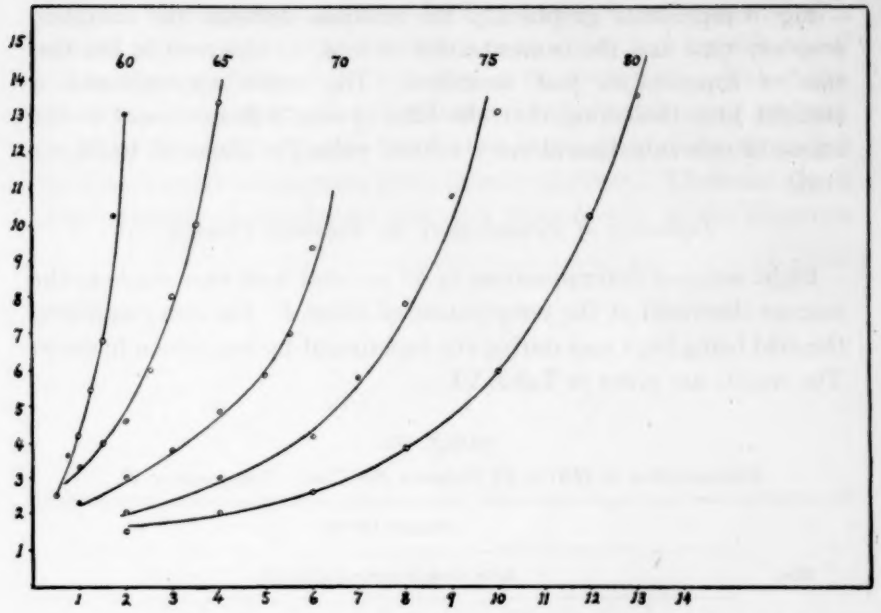

FIG. 2. Graphs for the second set of determinations with No. 25 wires. Otherwise as in Fig. 1. The circles represent the averages of Table V.

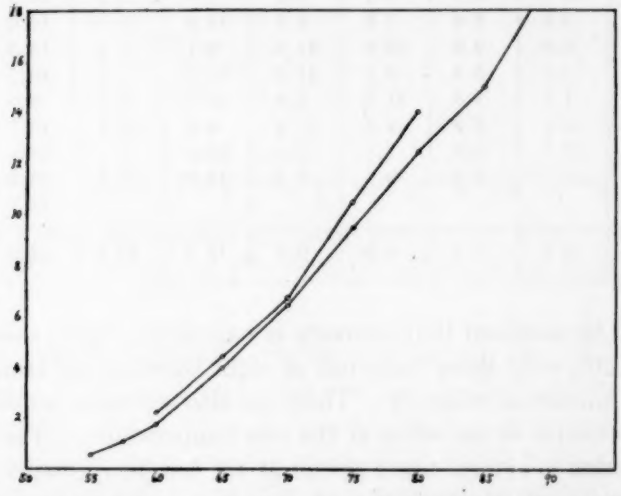

FIG. 3. Relation between complete recovery time and concentration of acid. Ordinates are times (minutes) required for recovery of complete transmissivity at 20°; abscissæ are concentrations (volumes per cent of 1.42 HNO_3). The right hand curve represents the earlier (data of Table II), the left hand the later set of determinations.

Fig. 3 represents graphically the relation between the complete recovery time and the concentration of acid, as observed in the two sets of experiments just described. The curve approximates a straight line, indicating that the time is nearly proportional to the excess of concentration above a critical value (of about 53 to 54 per cent).

Influence of Temperature on Recovery Process.

Eight series of determinations in 65 per cent acid were made in the manner described at the temperature of about 3°, the dish containing the acid being kept cool during the experiment by immersion in snow. The results are given in Table VI.

TABLE VI.

Concentration of HNO_3, 65 Volumes Per Cent. Temperature 3°.

Wire.	Distance traveled. Intervals since previous activation.							
	2 min.	4 min.	6 min.	8 min.	10 min.	12 min.	14 min.	16 min.
	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.
A	4.1	4.9	6.1	9.2	8.3	19.2	16.8	Full.
B	3.8	5.8	7.0	8.8	13.4	10.3	14.6	8.6
C	4.9	9.0	10.6	11.5	9.7	17.3	13.3	16.8
D	2.2	3.3	9.1	11.2	11.4	11.9	16.2	8.4
E	1.7	5.5	11.3	5.8	14.8	10.7	9.6	19.3
F	3.1	3.9	9.3	11.4	6.4	10.1	13.1	14.8
G	3.5	3.9	7.7	8.6	11.6	11.7	Full.	Full.
H	5.5	5.2	9.8	7.5	14.7	12.6	14.4	
							13.7	
Average.	3.5	5.2	8.9	9.2	11.3	13.0	14.0	

It will be observed that recovery is four or five times slower at 3° than at 20°, only three cases out of eight showing full transmission after 16 minutes of recovery. There are also several other differences in the behavior of the wires at the two temperatures. The speed of transmission is several times slower at the low temperature; this retardation is perfectly distinct to the eye, but as yet no exact measurements of velocity have been made. The local reaction shows a less

vigorous effervescence and lasts for 5 or 6 seconds, as compared with about 1 second at 20°; *i.e.*, the return to passivity is delayed in about the same proportion as the recovery of transmissivity. The disappearance of the deposit of dark oxide is also much more gradual. In partly recovered wires the retardation in the speed of the activation wave as it nears its stopping point is more distinct. There are also a larger number of apparently arbitrary irregularities in the distances

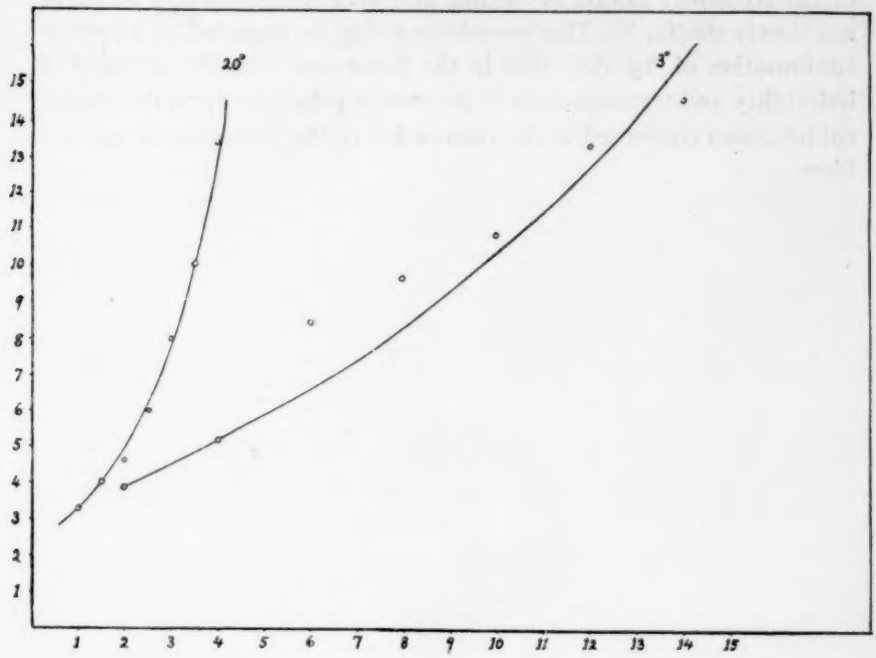

FIG. 4. Curves showing rates of recovery in 65 per cent acid at the two temperatures, 20° (Table V), and 3° (Table VI). Ordinates are distances (cm.), abscissæ, times as before.

traveled; thus Wires B and D transmitted for only about 8.5 cm. after 16 minutes in the acid, and other reversals in the expected order of the figures are seen in Wires A, C, E, F, and H. Apparently the conditions (irregular local circuits, etc.) which interfere with transmission are more effective when the local chemical processes are slower. The points representing the average transmissions at the several in-

tervals group themselves with fair regularity into a curve of the usual type but of more gradual slope (Fig. 4).

The characteristic temperature coefficient shown (Q_{10} = 2–3) is evidently an expression of the dependence of repassivation and recovery of transmissivity upon chemical reactions occurring at the metallic surface. These reactions rebuild the surface film and restore it to its original condition. The temperature coefficients of the duration of the refractory period in cardiac and skeletal muscle and in nerve are closely similar.[15] This resemblance may be regarded as a further confirmation of the view that in the latter case also the recovery of irritability and transmissivity is dependent primarily upon the chemical processes concerned in the restoration of the protoplasmic surface films.